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The link of asthma outcome with AMP responsiveness and airway pathology

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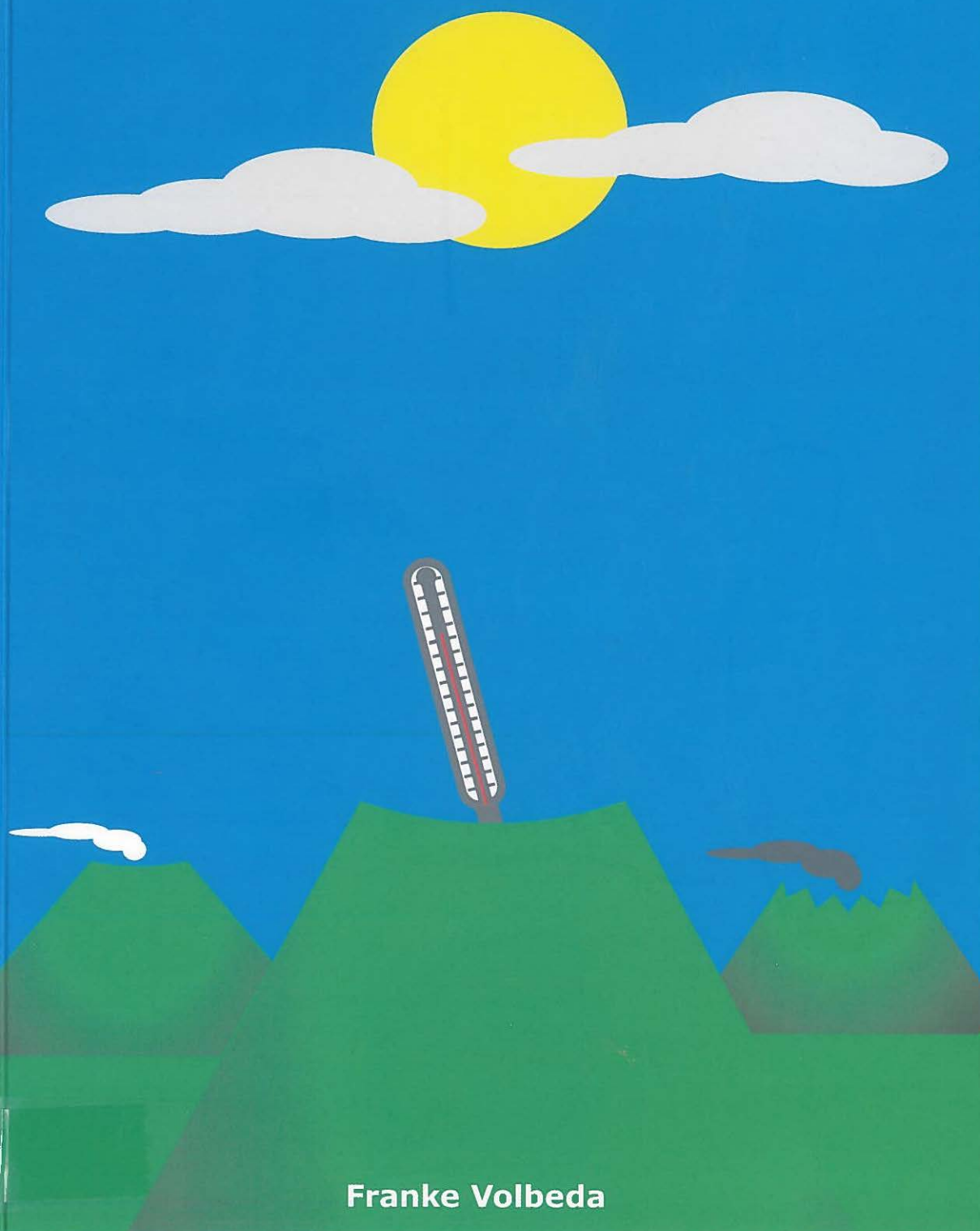
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Franke Volbeda

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Stellingen

behorende bij het proefschrift

The link of asthma outcome with AMP responsiveness and airway pathology

1. Complete remissie van astma bestaat (dit proefschrift)
2. Het fenotype van astma dient uitgebreid te worden met het AMP profiel (dit proefschrift)
3. Stoppen met roken lijkt ook vanuit pulmonaal oogpunt zinvol bij astma, maar geeft (nog) geen garanties voor de toekomst (dit proefschrift)
4. De kunst is om de eosinofiele inflammatie op tijd te ontdekken omdat dit geassocieerd is met versnelde achteruitgang van longfunctie (dit proefschrift)
5. Versterkte luchtweggevoeligheid voor AMP is een geschikte indicator en provocator voor eosinofiele inflammatie (dit proefschrift)
6. Na het bereiken van Well Controlled astma, lijkt er geen basis te zijn om nog voor Total Control te gaan (dit proefschrift)
7. Klinische controle van astma heeft een fundament gekregen (dit proefschrift)
8. Om klinisch verder te komen, gaan we met het onderzoek juist verder van de dagelijkse kliniek af
9. Open deuren dienen ook evidence based te zijn
10. Het verschil tussen stilstand en beweging is op een foto moeilijk te onderzoeken
11. Als de sleutel voor behandel succes zich in de handen van de patiënt bevindt, is het de uitdaging voor de dokter om het slot te wijzen





rijksuniversiteit
 groningen

**The link of asthma outcome with AMP
responsiveness and airway pathology**

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
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General Introduction

THE NATURAL COURSE OF ASTHMA

Asthma is a chronic inflammatory disorder of the airways associated with airway obstruction that is often completely reversible. Chronic asthmatic disease may lead to important morbidity and may have a large impact on quality of life. These complications are most likely the result of an inflammatory process in the airways underlying the clinical presentation of asthma. As a result of activation of inflammatory cells, an array of mediators and cytokines are produced and/or released, causing bronchoconstriction, vasodilatation, plasma exudation, mucus hypersecretion and activation of sensory nerves, all contributing to the clinical presentation of asthma. Simultaneously this ongoing inflammatory process may result in structural changes in the airways, such as subepithelial fibrosis, hyperplasia of airway smooth muscle cells, and angiogenesis. Ultimately these processes may lead to irreversible lung damage in some (but not all) asthmatics. This has indeed been found after long-term follow up of asthmatics, resulting in irreversible airflow limitation(1-4)

Next to deterioration of disease over time, there may also occur asthma remission over time. This can occur both in a subset of adult and childhood asthma (3;5), meaning that these patients have normal lung function, and no clinical symptoms while not using asthma medication. This state has been defined as clinical or symptomatic asthma remission(4-7). However, since bronchial hyperresponsiveness is still present in the majority of patients with clinical or symptomatic remission(4-7), this suggests that the underlying disease process is not yet cured. This is supported by the observation that the bronchoalveolar lavage (BAL) fluid contains increased levels of eosinophils in some asthmatic children without current symptoms and medication for the last 12 months, when compared to healthy controls(8). Furthermore, higher numbers of eosinophils, T cells, mast cells and higher expression of IL-5 were found in the airway mucosa of patients with clinical remission, when compared to healthy controls(9).

Given the persistence of bronchial hyperresponsiveness and elevated markers of pulmonary inflammation, despite the asymptomatic status in this group of patients, the definition of complete asthma remission has been introduced. This definition also includes absence of bronchial hyperresponsiveness as an additional criterium to the asymptomatic status(4;5). So far no studies have investigated pulmonary inflammation in this subset of asymptomatic patients and assessed if these subjects are really "cured" from their asthma.

DETERMINANTS FOR PROGRESSION OR REMISSION OF ASTHMA

Still little is known with respect to risk factors that determine progression or remission of asthma. It has been suggested in several studies that progression to persistent airway obstruction is more closely related to the duration of asthma than to age itself(1;2;10). Previous studies have revealed multiple risk factors for an accelerated FEV₁ decline (15-50 ml/year) in asthma: low baseline lung function (FEV₁ % predicted)(11-13), less reversibility to β_2 -agonists(2;10), more severe bronchial hyperresponsiveness(12;14), smoking(15), increased mucus production(16), male gender(17), and smoking (16)

The pathological processes underlying the accelerated FEV₁ decline in asthma are unknown. Several suggestions for the underlying pathological process come from cross-sectional studies demonstrating an association between the above described risk factors and markers of airway remodeling and/or inflammation. For example, low baseline lung function has been associated with increased airway wall thickness(18). A more severe BHR has been associated with thickening of the subepithelial basement membrane(19), and with subepithelial fibrosis(20). Increased mucus production has been associated with hyperplasia and hypertrophy of epithelial goblet cells(21) and with increased presence of submucosal mucous glands(21). Also, an association has been shown between accelerated FEV₁ and higher blood eosinophil numbers and decline in adult asthma patients(22).

Not all asthmatics show this excess decline. Panhuysen et al investigated which factors contribute to outgrowing of asthma, which may occur in up to 25 % in the group of adult

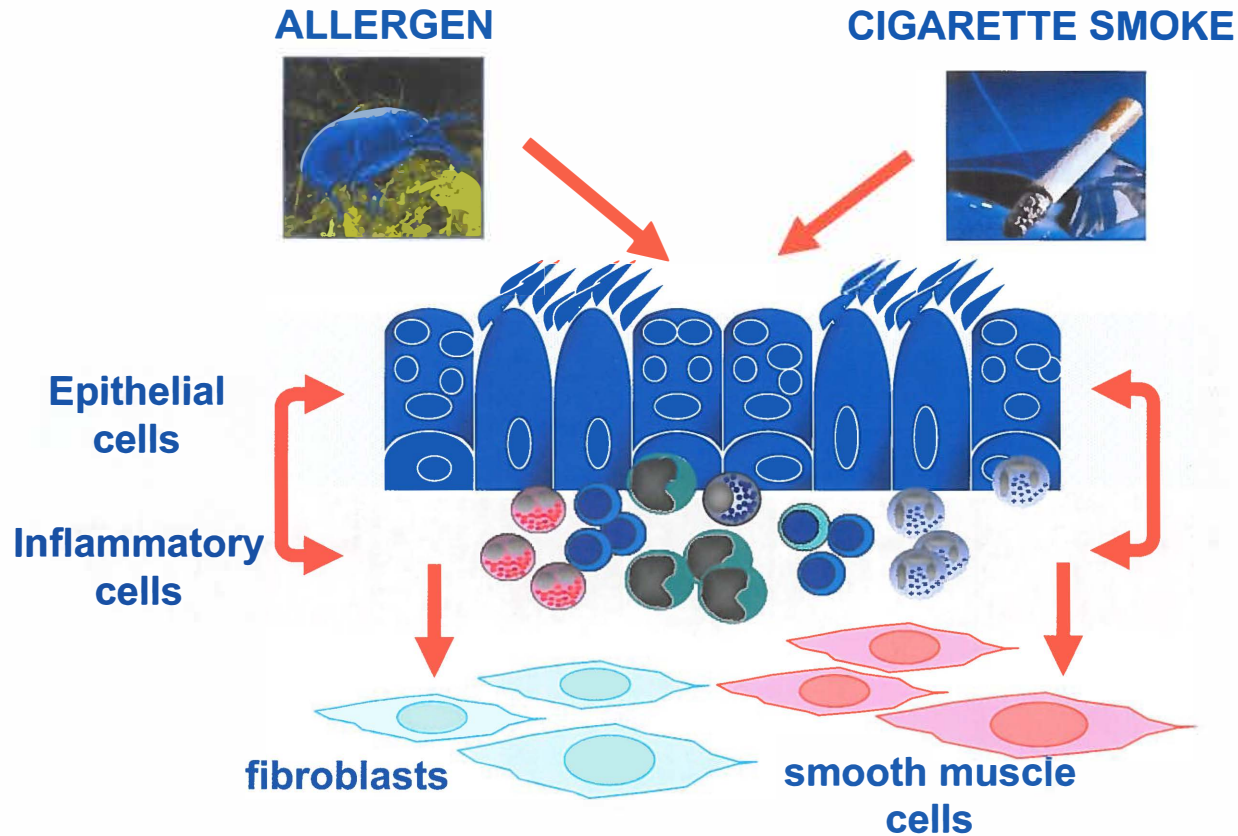
asthma patients(5). Disappearance of asthma was associated with younger age and with less severe airway obstruction at first testing(5). In line, disappearance of airway hyperresponsiveness was associated with younger age and with both less severe airway obstruction at first testing and with a shorter untreated period of asthma(5). Grol et al investigated the outcome of asthma in children, and confirmed that a better lung function in childhood was associated with remission of asthma at young adulthood(3).

The above mentioned risk factors for progression of asthma suggest that an accelerated FEV₁ decline in asthma indeed results from ongoing inflammatory and remodeling processes in the airways. Furthermore, risk factors for remission of asthma support the hypothesis that early (sub)epithelial and inflammatory factors in the airway wall already determine whether patients may outgrow the disease. Probably, a combination of environmental and genetic risk factors plays a role in the ongoing process of airway inflammation and remodeling, consequently leading to the clinical progression or remission of asthma. More studies are needed to disentangle the causative factors. For an accurate assessment of the (lack of) decline in FEV₁, it is very important to take the three phases in the development of FEV₁ into account, i.e. initial growth, followed by a plateau phase, and after the age of \pm 30 years decline of FEV₁(23;24).

AIRWAY INFLAMMATION

Asthmatic airway inflammation is characterised by a complex interplay of resident cells (epithelial and dendritic cells, fibroblasts, nerves, endothelial cells) and inflammatory cells (eosinophils, mast cells, macrophages, T-lymphocytes). Asthmatic inflammation contributes to the airway obstruction that is caused by inhalation of allergens. Allergen inhalation typically induces both an early response, with airway obstruction within 10 minutes, and a late response that usually is associated with airway obstruction after 8-24 hours. The early asthmatic reaction following inhaled allergen provocation is mast-cell dependent. Mast cells secrete mediators like histamine, prostaglandin D₂, and leukotriene C₄, which are capable of inducing bronchoconstriction, mucus secretion, and mucosal edema. Mast cells in asthma localize in the airway smooth muscle (ASM), airway mucous glands, and bronchial epithelium. In these locations, mast cells may contribute to the development of ASM hypertrophy and hyperplasia, may facilitate the ongoing immunologic response (antigen presentation, Th2 differentiation and IgE synthesis), and may have consequences on epithelial function. Also, in post-mortem studies, mast cells have been associated with mucous gland hyperplasia and the mucus gland secretion(25).

It is suggested that a structurally and functionally defective airway epithelium underlies abnormal responses to inhaled stimuli in asthma. These abnormal responses enhance signalling between the airway epithelium and underlying structural and immune cells. Eventually, this promotes a microenvironment that facilitates allergic sensitization, supports different types of inflammation, and predisposes the airways to exacerbations(26). The inflammatory cascade is thought to start with the presentation of an allergen by a dendritic cell to Th2 lymphocytes. This activates Th2 lymphocytes resulting in an asthma-characteristic release of IL-4 and IL-5 cytokines. This ultimately leads to allergen-specific IgE production by B cells and the recruitment, proliferation and maturation of the asthma-specific effector cell, the activated eosinophil. Activated eosinophils may release mediators like eosinophil-derived neurotoxin (EDN), major basic protein (MBP) and eosinophil cationic protein (ECP) that are cytotoxic and damaging to the airway epithelium. This damage to the airway epithelial may initiate a chronic inflammatory state of the airway wall. Th1 lymphocytes may contribute to chronicity and to the effector phase in allergic disease. Th1 lymphocytes increase IFN- γ and TNF- α levels which promote activation and apoptosis of epithelial cells associated with epithelial shedding in asthma(27).



Chronic asthma is characterized by enhanced epithelial-mesenchymal communication in response to inhaled stimuli. The continuous release of proinflammatory products and growth factors eventually leads to airway remodelling

Chronic airway inflammation underlies the structural and irreversible changes of the airways. Several immunological mechanisms may contribute to the consolidation of airway inflammation(28;29). The bronchial epithelium of asthmatics seems to become fragile and to lose its protective properties. Increased expression of CD44 suggests that the bronchial epithelium transforms into a chronic repair phenotype with enhanced presentation of epidermal growth factor ligands to their receptors. Eventually, the bronchial epithelium becomes a continuous source of proinflammatory products as well as growth factors that drive airway wall remodeling(29).

AIRWAY REMODELING

Morphometric studies in post-mortem or resected lung specimens have confirmed that the airway walls of subjects with fatal and non-fatal asthma are thicker than those of non-asthmatics. Other studies have shown a significant correlation between increased airway wall thickness and irreversible airway obstruction{Boulet, 1995 221 /id}. The structural changes that are associated with increased airway wall thickness and chronic airway obstruction are:

- Goblet cell hyperplasia and/or epithelial metaplasia(21)
- Thickened lamina reticularis or subbasement membrane(20;30;31)
- Submucosal collagen deposition(32)
- Smooth muscle hyperplasia and hypertrophy(33;34)
- Mucous gland hyperplasia(35)
- Increased vascularity(36)
- Small airway and parenchymal changes(37;38)

ASTHMA AND SMOKING

The prevalence of cigarette smoking among adults with asthma is surprisingly high. In a 15-year follow-up study in Denmark the prevalence of current smoking in subjects with asthma (mean age 52 years) was 71%, while in subjects without asthma this was 62% ($p < 0.001$)(16). In The Netherlands, the prevalence of cigarette smoking in a cohort of young asthmatics with a mean age of 24.7 years was 33%(39) which is comparable to the general population at that time.

The clinical outcome of asthma is negatively affected by cigarette smoking. Asthmatic smokers have more severe asthma symptoms than non-smoking asthmatics(40), a higher frequency of asthma attacks(16), more hospital admissions(41;42), accelerated lung function decline(16;16) and increased asthma mortality(43).

Cigarette smoking has negative effects on the lung in non-asthmatic subjects, causing increased airway inflammation, i.e. increased neutrophil, macrophage and CD8+ lymphocyte numbers in BAL(44-47) and increased inflammatory cell numbers in the bronchial wall, such as neutrophils, eosinophils, macrophages and mast cells(45;47;48). Moreover, cigarette smoking has been associated with features of increased remodeling, such as increased tenascin and laminin deposition under the basement membrane, in non-asthmatic subjects(48).

At present, the underlying pathological processes of smoke-induced deterioration of asthma are largely unknown. As smokers are often excluded in asthma studies to prevent co-existence of chronic obstructive pulmonary disease, only a few studies have presented results with respect to the effects of smoking on asthmatic inflammation. These few studies showed that cigarette smoking has an impact on inflammation by increasing the number of neutrophils(49;50) and IL-8 levels(50) in sputum and by increasing bronchial metaplasia, neutrophil elastase and IFN- γ and intra-epithelial IL-8 in biopsies(51;52). Also white blood cell numbers are increased in asthmatic smokers(49;50).

MARKERS OF PULMONARY INFLAMMATION

Bronchial hyperresponsiveness tests are regarded to be markers of airway inflammation. Traditionally, bronchial hyperresponsiveness is measured with methacholine or histamine; both are direct stimuli since they exert their effect directly on airway smooth muscle cell receptors. One study investigated a treatment strategy in which changes in treatment were based on regularly performed hyperresponsiveness tests to methacholine on top of the Global Initiative for Asthma (GINA) guidelines(53). As a consequence of this strategy the dose of inhaled corticosteroids was almost doubled in the hyperresponsiveness arm versus GINA arm. As expected, this increased dose of inhaled corticosteroids was associated with clinically relevant improvements in respiratory symptoms, with less airway obstruction, with less hyperresponsiveness to methacholine, and with decreased airway inflammation and airway remodeling. A limitation to this study protocol may be that bronchial hyperresponsiveness to methacholine has a limited sensitivity to inflammatory changes after adjusting the dose of inhaled corticosteroids. The increase in the doubling dose of methacholine was only 1.1 in the hyperresponsiveness arm after 2 years of treatment. This limited sensitivity probably contributes to the higher dose of inhaled corticosteroids in the hyperresponsiveness arm and may limit the option of a step down regime by titrating corticosteroid doses.

Another stimulus to measure bronchial hyperresponsiveness is Adenosine 5'-MonoPhosphate (AMP). AMP is an indirect stimulus, since it is thought to act via the release of inflammatory mediators from mast cells. It has been suggested that the response to AMP better reflects eosinophilic inflammation in asthma than the response to direct stimuli. The level of PC₂₀ AMP was predominantly predicted by the percentage of sputum eosinophils in a cross-sectional study(54). In addition, the extent of corticosteroid-induced improvement in PC₂₀ AMP was solely related to the level of reduction in airway inflammation as reflected by reduced numbers of sputum eosinophils, lymphocytes, epithelial cells, and exhaled NO(55). Furthermore, bronchial hyperresponsiveness to AMP has been shown to respond more rapidly to treatment with inhaled corticosteroids than a challenge with a direct stimulus, with changes in PC₂₀ AMP values already detectable after 1 week of treatment and returning to near-baseline levels after 1-week treatment discontinuation. In contrast, significant changes in PC₂₀ methacholine are only detectable after 4 weeks of treatment and no significant changes are found after 4 weeks treatment discontinuation(56).

Measuring exhaled Nitric Oxide (NO) has been found useful in assessing and monitoring of airway inflammation. Previous studies have found an association between exhaled NO levels and asthma severity(57;58). Also treatment with inhaled corticosteroids leads to lower exhaled NO levels(59). However, in the same study, exhaled NO did not correlate with airway mucosal eosinophilia and conventional markers of asthma control(59). A recent review of 5 studies showed that treatment guided algorithms based on exhaled NO did not reduce the frequency of asthma exacerbations, in contrast to algorithms based on sputum eosinophils (60). Only one study with exhaled NO guided therapy showed that the maintenance ICS dose could be reduced according to NO levels (61).

As mentioned above, induced sputum has also been used to monitor asthma inflammation during treatment. Green et al. measured eosinophils in induced sputum and adjusted inhaled corticosteroid levels according to eosinophil numbers, targeting a level of 1 and 3%. Sputum based medication adjustments led to fewer (severe) asthma exacerbations and hospital admissions compared to the control group. In both groups, the doses of inhaled corticosteroids used were similar(62). In another study, data on asthma exacerbations was compared between treatment guided on eosinophils in induced sputum and on clinical data. With sputum guided treatment the time to the first exacerbation was longer (by 213 days), the relative risk ratio for an asthma exacerbation was lower (by 49%), and the number of exacerbations needing prednisone was reduced (5 versus 15)

when compared to the control group. This strategy did not improve the frequency of non-eosinophilic exacerbations, which were the most common exacerbations(63). Recently, the same group showed, in a pilot study of 20 patients, that a treatment strategy aiming at normalising sputum eosinophils also reduced mucosal inflammatory cells and MUC5A expression in bronchial biopsies in patients with mild to moderate asthma. However, no effects on airway remodeling were found as measured by subepithelial collagen deposition(64).

ASSESSING CONTROL OF ASTHMA

The GINA guidelines of 2006 emphasized the importance of asthma control, focusing on the level of impairment, the patient's future risk of exacerbations, loss of lung function and drug side-effects.

The GOAL (Gaining Optimal Asthma control) study in 2004 aimed at achieving optimal control by applying strictly the aims of asthma treatment as defined by GINA in 2002. Optimal-control and well-control of asthma in this study were composite measures based on respiratory symptoms at day and night, use of rescue medication, peak expiratory flow rate, exacerbations, emergency visits and treatment-related adverse effects(65). These variables are easy to measure and applicable worldwide. Indeed many patients without control of their asthma improved to well-control or optimal-control levels after stepwise increase in the dose of fluticasone or fluticasone combined with salmeterol(66).

Several questionnaires have been developed to measure asthma control. One of the questionnaires is the 7 items asthma control questionnaire (ACQ), which has been validated in a 9-week observational study in 50 adults with symptomatic asthma(67). The reliability of the ACQ was very high in patients with stable asthma (intra class correlation coefficient 0.90). The questionnaire responded significantly to changes in asthma control. Furthermore, high correlations were shown with asthma related quality of life (AQLQ questionnaire)(68), general health status, and other asthma symptoms. A drawback of the ACQ is that it requires measurement of peak expiratory flow, which could limit its use because a peak flow meter may not always be available or may not be properly used. To exclude the need for peak flow measurements, the short version of the ACQ was introduced which showed similar reliability(69).

More recently the asthma control test (ACT) was introduced as an alternative questionnaire for quick assessment of uncontrolled asthma(70). The ACT scores 5 items and an overall agreement between ACT and the specialist's rating ranged from 71% to 78% depending on the cut-off points used(70). Longitudinally, responsiveness of the ACT to changes in asthma control and lung function was demonstrated with significant correlations between changes in ACT scores and changes in specialists' ratings ($r=0.44$, $P<.001$), ACQ scores ($r=-0.69$, $P<.001$), and percent predicted FEV1 values ($r=0.29$, $P<.001$)(71). An ACT score of 19 or less provided optimum balance of sensitivity (71%) and specificity (71%) to detect uncontrolled asthma(71). Thus, the ACT seems to be a useful tool in the clinical setting to identify patients with uncontrolled asthma and to follow patients' progress with treatment.

Only a few studies have investigated associations between assessment of the level of asthma control by questionnaires and the severity of airway inflammation. Quaedvlieg et al showed that higher numbers of sputum eosinophils and more severe bronchial hyperresponsiveness to methacholine, but not higher exhaled NO levels, were associated with uncontrolled asthma as scored with the ACQ(72). Other studies found correlations between asthma questionnaires and exhaled NO (73) and inflammatory markers in induced sputum(74;75).

So far, no studies have investigated the relationship between the level of asthma control as assessed by questionnaires and airway inflammation in bronchial biopsies. Such studies are highly needed to investigate whether there is a direct link between subjective markers of asthma control and objective markers of airway inflammation and airway remodeling.

Chapter 1

AIMS OF THE STUDIES

1. To investigate if airway inflammation and remodeling are absent in asthma patients meeting the criteria of complete asthma remission (Chapter 2 and 3).
2. To investigate differences in airway inflammation and remodeling between adenosine-5'-monophosphate responsive and unresponsive asthmatics (Chapter 4).
3. To investigate differences in airway inflammation and remodeling between remission and progression of asthma (Chapter 5).
4. To investigate the underlying pathological process of smoke-induced deterioration of asthma (Chapter 6.)
5. To investigate the relationship between clinical markers of asthma control with both direct and indirect markers of airway inflammation and remodeling (Chapter 7).

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Chapter 1

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Can AMP Induce Sputum Eosinophils, Even in Subjects with Complete Asthma Remission?

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ABSTRACT

Background: The definition of "clinical asthma remission" is based on absence of symptoms and use of medication. However, in the majority of these subjects airway inflammation is still present when measured. In the present study we investigated whether "complete asthma remission", additionally defined by the absence of bronchial hyperresponsiveness (BHR) and the presence of a normal lung function, is associated with the absence of airway inflammation.

Methods: Patients with a former diagnosis of asthma and a positive histamine provocation test were re-examined to identify subjects with complete asthma remission (no asthma symptoms or medication, PC20 histamine > 32 mg/ml, FEV₁ > 90% predicted). Patients with PC20 histamine ≤ 32 mg/ml were defined as current asthmatics and were divided in two groups, i.e. asthmatics with and without BHR to adenosine 5'-monophosphate (AMP). Sputum induction was performed 1 week before and 1 hour after AMP provocation. Sputum induction and AMP provocation were previously shown to be sensitive markers of airway inflammation.

Results: Seven patients met criteria for complete asthma remission. Twenty-three were current asthmatics, including twelve without hyperresponsiveness to AMP. Subjects with complete asthma remission showed no AMP-induced sputum eosinophilia (median (range) 0.2 (0 – 4.6)% at baseline and 0.2 (0 – 2.6)% after AMP). After AMP, current asthmatics had a significant increase in sputum eosinophils (0.5 (0 – 26.0)% at baseline and 2.6 (0 – 32.0) % after AMP), as had the subgroup of current asthmatics without hyperresponsiveness to AMP (0.2 (0 – 1.8)% at baseline and 1.3 (0 – 6.3)% after AMP).

Conclusions: Subjects with complete asthma remission, in contrast to subjects with current asthma, do not respond with eosinophilic inflammation in sputum after AMP provocations. These data lend support to the usefulness of the definition of complete asthma remission.

BACKGROUND

Asthma symptoms may diminish over time in a subgroup of patients and sometimes even disappear completely. The loss of asthma symptoms in the absence of need for pulmonary medication has been defined as "clinical or symptomatic asthma remission" (1-4). However, the majority of patients with clinical or symptomatic remission still shows bronchial hyperresponsiveness (BHR) (1-4), suggesting that the disease asthma is not yet cured.

This is supported by the observation that the broncho alveolar lavage (BAL) fluid contains increased levels of eosinophils in some asthmatic children with clinical asthma remission, when compared to healthy controls (5). Furthermore, higher numbers of eosinophils, T cells, mast cells and expression of IL-5 were found in the airway mucosa of patients with clinical remission than in healthy controls (6).

Given the persistent presence of airway inflammation and BHR despite the asymptomatic status, it is likely that a more strict definition is required to signify whether an individual really is "cured" from asthma and can be regarded to have complete remission. The definition of clinical asthma remission has therefore previously been extended to "complete asthma remission", also including absence of BHR and normal lung function ($FEV_1 > 90\%$ predicted) (1,4). A 25-year follow up study of 181 asthma patients, initially aged 13-44 years, all diagnosed with a positive bronchial hyperresponsiveness test to histamine and clinical asthma symptoms according to ATS criteria, showed a prevalence of clinical remission and complete remission of 40% and 11% respectively by the age of 38-69 years (1). In another follow up study, 119 allergic asthmatic children were retested after 30 years and 52% were in clinical remission and 22% in complete asthma remission (4). To our knowledge, it has not been investigated so far whether asthmatic airway inflammation is objectively absent in this subgroup of subjects with complete asthma remission.

We have previously shown that adenosine 5'-monophosphate (AMP) provocation induces sputum eosinophilia in asthma patients with and without inhaled steroid use (7). We considered this as an interesting tool to investigate airway inflammation, because the recruitment process of eosinophils after an AMP challenge indicates the presence of an active inflammatory response.

The present study was set up to investigate whether such an inflammatory response indeed is absent and cannot be induced in subjects with complete asthma remission. To this aim we compared inflammatory cells 1 week before and 1 hour after AMP provocation in induced sputum from subjects with complete remission and from patients with current asthma. Since not all asthmatics are responsive to AMP, we divided these subjects in an AMP responsive and a non-responsive group. These subgroups enabled us to additionally study the dose-response effect of AMP on sputum eosinophil numbers.

METHODS

SUBJECTS

Non-smoking asthma patients aged between 18 and 70 years, without oral or inhaled corticosteroids were recruited. All patients originated from research cohorts investigated earlier by our research group and all had a doctor's diagnosis of asthma and a documented PC_{20} histamine ≤ 32 mg/ml in the past (4;8-10). All patients had to be able to expectorate sputum after inhalation of hypertonic saline. In order to compare the effects of the highest cumulative dose of AMP (639.99 mg) on the influx of inflammatory cells in induced sputum, current asthma patients were divided in those with a negative and a positive AMP provocation test. Current asthma in these patients was proven by a positive bronchial hyperresponsiveness test to histamine. Patients were considered to have no asthma symptoms when they answered negatively on questions regarding cough and sputum in wintertime, dyspnea, wheeze and asthma attacks in the last three

years. The study protocol was approved by the local medical ethics committee; all participants gave their written informed consent.

Patients were assigned to 3 different groups according to the following criteria:

- Complete asthma remission: former diagnosis of asthma, PC₂₀ AMP > 320 mg/ml and PC₂₀ histamine > 32 mg/ml, FEV₁ % predicted > 90%, no asthma symptoms, no asthma medication.
- Current asthma with a negative PC₂₀ AMP: former diagnosis of asthma, PC₂₀ AMP > 320 mg/ml, PC₂₀ histamine ≤ 32 mg/ml.
- Current asthma with a positive PC₂₀ AMP: former diagnosis of asthma, PC₂₀ AMP ≤ 320 mg/ml.

STUDY DESIGN

Patients visited the hospital twice. At the first visit lung function, blood collection and sputum induction were obtained. The second visit followed after 1-2 weeks and included AMP provocation test and sputum induction 1 hour after the final dose of AMP. In patients with a negative AMP provocation (dose AMP > 320 mg/ml) bronchial hyperresponsiveness to histamine was measured > 1 week later. To facilitate comparison with historical data on bronchial hyperresponsiveness to histamine, the maximum provocative dose of histamine in the present study was also set at 32 mg/ml (with 30-seconds tidal breathing method, a dose that is comparable with 8 mg/ml in the 2-minute tidal breathing method).

QUESTIONNAIRE

The Dutch version of the British Medical Research Council's standard questionnaire was used(11). Patients were considered asymptomatic if they answered negatively on questions regarding cough, sputum, dyspnea, wheeze and asthma attacks

LUNG FUNCTION

FEV₁ was measured with a calibrated water-sealed spirometer according to standardized guidelines(12;13). Reversibility of the FEV₁%predicted was measured after administration of 400 µg of salbutamol. Provocation tests were performed with a method adapted from Cockcroft and coworkers(14;15). After 2-min tidal breathing and an initial nebulized saline challenge, subjects inhaled doubling concentrations AMP (0.04 to 320 mg/ml) at 5-min intervals. Bronchial hyperresponsiveness to histamine was tested as reported previously(8), using 30-seconds tidal breathing and doubling concentrations ranging from 0.13 to 32 mg/ml.

SPUTUM INDUCTION AND SPUTUM PROCESSING

Sputum was induced by inhalation of hypertonic saline aerosols as previously described(13). Hypertonic saline (5%) was nebulized over 3 consecutive periods of 5 min. Whole sputum samples were processed according to the method of Fahy and colleagues with some modifications(13;16). Sputum cell cytopins were stained with May Grünwald Giemsa (MGG) and cell differentials from in total 600 viable, non squamous cells were assessed in a blinded fashion. Sputum was not scored if the percentage of squamous cells was > 80 percent or the total number of non-squamous cells was < 600. Number of sputum drop-outs because of > 80 % squamous cells were 6 at baseline and 0 after AMP, drop outs because of < 600 non-squamous cells were 2 at baseline and 4 after AMP. Additionally, no sputum could be induced in 8 patients at baseline and 6 after AMP.

HISTAMINE ELISA

A histamine ELISA was purchased from IBL (Hamburg, Germany) for quantitative detection of histamine in sputum supernatant. All reagents were provided in the kit. The protocol was as follows: the acetylated samples, controls and standards are pipetted into a 96-wells plate. After adding enzyme conjugate and histamine antiserum the plate was incubated for 3 hours on an orbital shaker. After washing TMB substrate solution was added to each well and incubated for 20 minutes. The substrate reaction was stopped and the optical density was measured at 450nM.

ALLERGIC PARAMETERS

The concentration of eosinophilic cationic protein (ECP) in sputum was measured using a fluoroenzyme immunoassay (ImmunoCAP ECP, Pharmacia, Uppsala, Sweden). Total serum IgE (IU/L) was measured by a solid-phase immunoassay (VIDAS total IgE kit, BioMérieux, Marcy l'Etoile, France). The Phadiatop screening test was performed on the ImmunoCap system according to the instructions of the manufacturer (Phadia AB, Sweden). Results were presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Positive Phadiatop was defined as patient serum/control serum >1.

STATISTICS

All analyses were performed using SPSS (version 16.0; SPSS Inc, Chicago, IL, USA). Non-parametric tests were used for analysis. Wilcoxon signed rank test was used for paired testing within groups between measurements at baseline and after AMP provocation. Mann-Whitney U test was used for testing differences between groups. Two tailed p-values of < 0.05 were considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS

47 patients were enrolled in the study. 17 patients were excluded from analysis because they were not able to produce sputum both before and after AMP provocation or because the quality of sputum was too low to allow analysis. 3 of the excluded subjects had complete asthma remission and 14 current asthma. The included and excluded groups did not differ statistically regarding age, sex, FEV1 %pred, reversibility of FEV1, allergy and blood eosinophils. From the included patients, 7 patients met the inclusion criteria for complete asthma remission and were compared with 23 current asthma patients. Of these current asthmatics 11 had a positive PC20AMP (PC20 AMP \leq 320 mg/ml) and 12 had a negative PC20 AMP (PC20 AMP > 320 mg/ml, but a PC20 Histamine \leq 32 mg/ml) (table 1). FEV1 % predicted in subjects with complete asthma remission was significantly higher than in patients with current asthma. Atopy was most frequent in asthmatics with a positive PC20AMP, significantly more frequent than in current asthmatics with a negative PC20 AMP and subjects in complete asthma remission

TABLE I: PATIENT CHARACTERISTICS

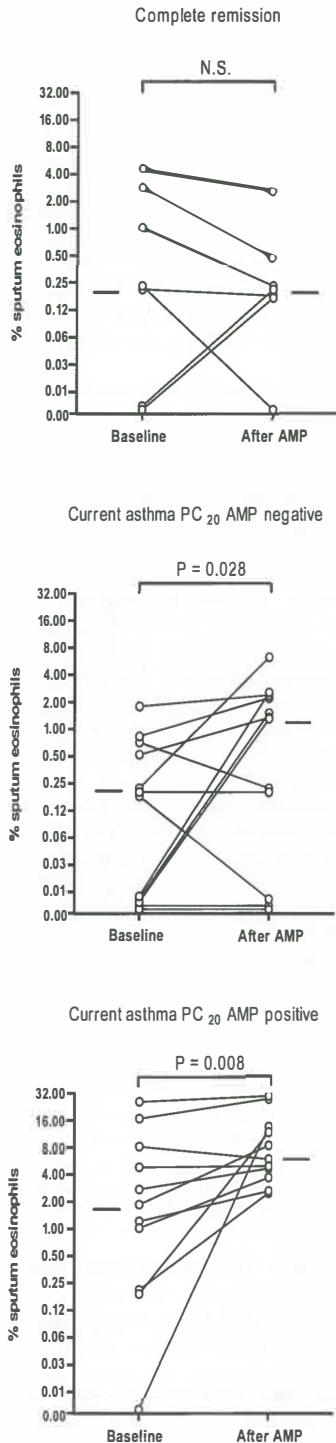
	Complete remission	Current asthma		
	(n=7)	Total group (n=23)	Negative PC ₂₀ AMP (n=12)	Positive PC ₂₀ AMP (n=11)
Sex (M/F)	2/5	14/9	6/6	8/3
Age (years)	53.0 (32-67)	48.0 (35-70)	47.5 (35-70)	48.0 (38 -62)
FEV ₁ (% predicted)	108.0 (102-144)*,††	97 (76-131)	96.2 (76.2 – 123)	96.5 (84.8 – 131)
PC ₂₀ AMP (mg/ml)	>320	>320 (0.02 – >320)	>320	12.0 (0.02 – 174)
Cumulative AMP dose (mg)	639.99	639.99 (0.02 –639.99)	639.99	39.99 (0.02 – 639.99)
PC ₂₀ Histamine (mg/ml)	>32.0	N.A.	16.9 (2.5 – 32.0)	N.A.
Reversibility FEV ₁ (%)	4.59 (1.76 – 9.72)	5.42 (-2.98- 26.7)	3.15 (-2.98 – 12.2)†	9.40 (1.76 – 26.7)
IgE (IU/L)	47 (7-166)	66 (11-558)	61 (14 – 246)	78 (11-558)
Positive Phadiatop (n (%))	3 (43)†	15 (65)	5 (42)†	10 (91)
Eosinophils blood (x10 ⁹ /L)	0.12 (0.09-0.24)	0.15 (0.03-0.35)	0.11 (0.03 – 0.35)	0.17 (0.07 – 0.25)

Values are medians (ranges), unless stated otherwise.

Definition of abbreviations: PC₂₀ AMP = provocative concentration of adenosine 5'monophosphate causing a 20% fall in FEV₁; reversibility FEV₁ = change in FEV₁, expressed as increase in percentage predicted normal value after 400 µg of Salbutamol; Positive Phadiatop = specific IgE's in patient serum/control serum >1; n = number; N.A. = not available.

* p ≤ 0.05 versus current asthma total group.

† p ≤ 0.05, †† p ≤ 0.01 versus current asthma PC₂₀ AMP positive.



SPUTUM DATA

COMPLETE ASTHMA REMISSION VERSUS CURRENT ASTHMA

At baseline, total sputum cell count, macrophage, neutrophil, lymphocyte and eosinophil differential counts were similar in all investigated groups (table 2). After AMP provocation, sputum eosinophils did not increase in subjects with complete asthma remission in contrast to patients with current asthma, resulting in a significantly higher percentage eosinophils post AMP challenge in the current asthma groups (figure 1). Similar trends were observed for sputum ECP, i.e. median (range) levels before and after AMP were 16.0 (2.5 – 170.0) and 14.8 (2.6 – 63.7), respectively, in the group with complete asthma remission and 36.3 (6.0 – 2467.0) and 47.3 (9.0 – 1628.0) in the group with current asthma. Histamine levels in sputum supernatant were comparable in the group with complete asthma remission and with current asthma. Median (range) levels before and after AMP provocation were 13.4 (2.8 – 29.8) ng/ml and 12.4 (4.0 – 137.0) ng/ml, respectively, in the group with complete asthma remission, and 9.8 (0.2 – 38.0) ng/ml and 8.4 (3.0 – 30.5) ng/ml in the group with current asthma.

AMP provocation had little effect on the other inflammatory cells. Analysis with absolute sputum cell numbers showed comparable results with sputum cell percentages, however data are not presented because of differences in total cell numbers between groups.

Figure 1
Changes in sputum eosinophil % from baseline to post-AMP provocation in subjects with complete asthma remission, current asthma patients with a negative AMP provocation and current asthma patients with a positive AMP provocation. Data are presented in a semi-log plot to optimize visualization of minor and major changes.

AMP POSITIVE VERSUS AMP NEGATIVE SUBJECTS WITH CURRENT ASTHMA

Allergy was more frequently present in current asthmatics with a positive than with a negative AMP provocation test (table 1). Sputum eosinophils increased significantly after AMP provocation in current asthma patients, even in those without AMP responsiveness (figure 1). However, the increase in sputum eosinophils after AMP provocation was significantly higher in AMP positive subjects (table 2). Also higher ECP levels were observed in AMP positive current asthma patients, i.e. median levels before and after AMP were 41.6 (11.9 – 262.0) $\mu\text{g/l}$ and 62.2 (15.2 – 467.0) $\mu\text{g/l}$, respectively, compared to 32.3 (6.0 – 2467.0) $\mu\text{g/l}$ and 37.8 (9.0 – 1628.0) $\mu\text{g/l}$ in AMP negative current asthma patients. Histamine levels in sputum supernatant were comparable in AMP positive and AMP negative subjects with current asthma. Median (range) levels before and after AMP were 11.0 (4.8 – 38.0) ng/ml and 9.1 (4.9 – 28.0) ng/ml, respectively, in the AMP positive group, and 9.5 (0.2 – 28.8) ng/ml and 8.3 (3.0 – 30.5) ng/ml in the AMP negative group.

DISCUSSION

To our knowledge, this is the first study investigating sputum inflammation in subjects meeting the criteria for complete asthma remission. Our definition of complete asthma remission also includes a normal lung function and absence of bronchial hyperresponsiveness in addition to the absence of asthma symptoms and medication, which was previously defined as clinical asthma remission. Sputum inflammation at baseline and the response after AMP provocation was compared between subjects with current asthma and complete asthma remission. Levels of sputum eosinophils at baseline and blood eosinophils were not significantly different between these groups. However, we demonstrate that AMP provocation increases the number of sputum eosinophils in current asthmatics, yet not in those with complete asthma remission. Similar trends were observed for ECP. This thus suggests that the latter group indeed has outgrown their asthma and eosinophils are not in a primed state.

The present study shows the usefulness of measuring hyperresponsiveness and FEV₁ in recognizing subjects with complete asthma remission. Several studies have shown that a clinical definition based on the absence of asthma symptoms and no use of asthma medication is insufficient to definitely assess that asthma is cured. Many of these subjects still show features of persistent asthma, such as presence of hyperresponsiveness and/or a low lung function(1-4) or ongoing airway inflammation(5;6). The concept of complete asthma remission has previously been introduced as an alternative to the definition of clinical asthma remission. Complete asthma remission is defined by the combined absence of asthma symptoms, asthma medication, airway obstruction, and bronchial hyperresponsiveness(1;4). In the present study, we investigated the concept of complete asthma remission in more detail by measuring inflammatory cells in induced sputum before and after AMP provocation in subjects meeting the criteria for complete asthma remission. The finding that sputum eosinophils do not increase significantly after AMP provocation in these subjects supports the hypothesis that they are free of airway inflammation. Indeed, eosinophils that are characteristic of asthma are not attracted to the airways upon provocation with a stimulus that does attract these cells in patients with persistent asthma, even when similar doses of AMP are being inhaled.

This study also demonstrates that sputum induction after AMP provocation gives more information than sputum induction at baseline alone. After all, the differences between asthma remission and current asthma would not have been recognized if only baseline sputum measurements had been compared, especially since all asthma patients had stable disease without using inhaled steroids. We can speculate about the underlying mechanisms that are responsible for the differences observed. Apparently the immunological mechanisms involved in recruiting eosinophils into the airway lumen differs between subjects with complete asthma remission and patients with current asthma. It is possible that immunologically primed mast cells may have

TABLE II: SPUTUM BEFORE AND AFTER AMP PROVOCATION

	Complete remission	Current asthma		
	(n=7)	Total group (n=23)	Negative PC ₂₀ AMP (n=12)	Positive PC ₂₀ AMP (n=11)
Total cells (x10⁶/ml)				
Baseline	0.6 (0.1 – 2.9)	0.3 (0.1 – 2.3)	0.4 (0.1 – 2.3)	0.3 (0.2 – 2.1)
After AMP	0.2 (0.1 – 1.4) [§]	0.3 (0.1 – 3.0)	0.4 (0.2 – 3.0)	0.3 (0.1 – 0.8)
Change	-0.5 (-1.5 – 0.0)	0.0 (-1.7 – 1.5)	0.1 (-1.1 – 1.5)	0.0 (-1.7 – 0.2)
Squamous cells (%)				
Baseline	23.8 (5.3 – 67.0)	21.3 (0.8 – 70.3)	19.2 (0.8 – 70.3)	21.3 (4.7 – 43.3)
After AMP	41.5 (9.0 – 49.0)	17.5 (0.5 – 64.5)	19.9 (0.5 – 64.5)	17.5 (1.7 – 24.5)
Change	14.0 (-26.2 – 22.2)	-2.0 (-25.8 – 27.0)	-1.1 (-16.5 – 27.0)	-2.0 (-25.8 – 10.6)
Neutrophils (%)				
At baseline	62.2 (18.9 – 77.6)	55.2 (19.8 – 94.5)	56.8 (20.7 – 94.5)	55.2 (19.8 – 93.6)
After AMP	48.1 (15.0 – 90.4)	59.0 (22.3 – 96.8)	71.3 (25.7 – 96.8)	52.7 (22.3 – 71.0)
Change	-3.9 (-37.9 – 58.1)	-3.2 (-35.1 – 68.0)	-0.4 (-29.5 – 68.0)	-5.1 (-35.1 – 25.4)
Macrophages (%)				
At baseline	35.8 (19.9 – 77.7)	32.7 (5.3 – 76.4)	40.0 (5.3 – 76.4)	32.7 (5.6 – 76.2)
After AMP	49.1 (8.9 – 84.2)	29.2 (3.0 – 72.5)	23.1 (3.0 – 72.5)	39.1 (12.6 – 61.2)
Change	11.2 (-56.7 – 41.3)	1.2 (-65.1 – 28.6)	0.0 (-65.1 – 28.6)	1.2 (-37.1 – 20.6)
Eosinophils (%)				
At baseline	0.2 (0.0 – 4.6)	0.5 (0.0 – 25.7)	0.2 (0.0 – 1.8) ^{††}	1.9 (0.0 – 25.7)
After AMP	0.2 (0.0 – 2.6) ^{*†††}	2.6 (0.0 – 32.0) ^{§§§}	1.3 (0.0 – 6.3) ^{†††, §}	5.9 (2.5 – 32.0) ^{§§}
Change	-0.2 (-2.3 – 0.2) ^{**††, §§}	1.4 (-2.3 – 13.7)	0.7 (-0.5 – 6.1) [†]	2.7 (-2.3 – 13.7)
Lymphocytes (%)				
At baseline	2.2 (1.4 – 7.3)	1.9 (0.0 – 6.9)	2.0 (0.2 – 6.9)	1.9 (0.0 – 5.9)
After AMP	0.3 (0.0 – 2.5) ^{*††, §, §}	1.3 (0.0 – 6.1)	0.8 (0.0 – 6.1)	1.7 (0.7 – 4.8)
Change	-1.8 (-7.0 – 0.7)	-0.2 (-4.9 – 4.8)	-0.4 (-4.2 – 3.8)	0.1 (-4.9 – 4.8)

Squamous cells were not included in the number of total cells and in the percentages of all other cell types
Values are medians (ranges).

* p ≤ 0.05, ** p ≤ 0.01 versus current asthma total group.

† p ≤ 0.05, †† p ≤ 0.01, ††† p ≤ 0.001 versus current asthma PC₂₀ AMP positive.

§ p ≤ 0.05, §§ p ≤ 0.01 versus current asthma PC₂₀ AMP negative.

§§ p ≤ 0.05, §§§ p ≤ 0.01, §§§§ p = 0.001 versus at baseline.

disappeared in subjects with complete asthma remission. Thus activation of the adenosine 2b receptor by AMP might not take place and neither the release of mast cell mediators. The loss of immunologically primed mast cells was not reflected by our data on histamine levels in sputum supernatant, histamine levels being similar in all groups. Bronchial biopsy studies are needed to further investigate the role of mast cells in complete asthma remission.

This study does not completely prove that our subjects with complete asthma remission fully have outgrown their asthma, as we did not compare results with healthy controls. Several studies suggest that a cut off value of 1% for sputum eosinophils can discriminate between current asthma and healthy controls(17-19). In our study, two subjects with complete asthma remission had > 1% sputum eosinophils, possibly indicating that asthmatic inflammation may still be present in the airways. However, only one subject in the complete asthma remission group had a sputum eosinophil percentage > 1% after AMP provocation. To our knowledge, no studies have investigated AMP induced sputum eosinophils in healthy controls, which clearly needs further study. Obviously, biopsy studies comparing subjects with complete asthma remission and healthy controls are needed to obtain a definite answer as to whether airway inflammation has disappeared entirely in subjects with complete asthma remission. Such studies might validate sputum induction after AMP provocation as a useful non-invasive tool to recognize patients who really have outgrown their asthma. Also longitudinal studies are needed to investigate if symptoms do not recur in patients suspected to be in complete asthma remission.

To compare the effects of the high cumulative doses of AMP on the influx of sputum inflammatory cells, we divided patients with current asthma in groups with and without a bronchoconstrictive response to AMP. The PC20 AMP negative group, that still had a proven bronchoconstrictive response to histamine, received exactly the same cumulative dose of AMP as the complete asthma remission group (with negative PC20 histamine). In contrast to subjects with complete asthma remission, this group of current asthma patients with a negative PC20 AMP showed a modest but significant increase in sputum eosinophils after AMP provocation. This result indicates that AMP has a differential effect on bronchoconstriction and bronchial inflammation in asthmatics with proven hyperresponsiveness to histamine.

An intriguing finding is the discrepancy between the presence of a positive PC20 histamine and absence of PC20 AMP in current asthmatics. We realize that the cut off values of 320 mg for PC20 AMP and 32 mg/ml for histamine are arbitrary and not objectively based on the presence or absence of asthmatic airway inflammation. Nevertheless, it may well be that current asthmatics with a positive PC20 AMP represent a different subset of asthma patients. Indeed, significant differences between the two groups are present in our study. Despite a lower cumulative dose of AMP, those with PC20 AMP had significantly more sputum eosinophilia than those without a PC20 AMP and similar trends were observed with sputum ECP. In addition, reversibility to salbutamol and the presence of specific IgE in serum were significantly higher in the asthma group with a positive PC20 AMP. A similar association between AMP sensitivity and atopic status has been described in an earlier study(20). It would be of interest to follow the asthmatics with a negative AMP test to assess whether they will develop clinical asthma remission. In other words it might represent an intermediate state between full blown asthmatic inflammation and absence of inflammation.

CONCLUSIONS

We conclude that our results suggest that the definition of complete asthma remission is valid, since sputum eosinophils do not increase after AMP provocation. This is in contrast with the increase in sputum eosinophils in current asthma that occurs even in the absence of a bronchoconstrictive response to AMP.

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Persisting Remodeling and Less Airway Wall Eosinophil Activation in Complete Remission of Asthma

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ABSTRACT

Rationale: Asthmatics may outgrow symptoms despite not using treatment, while others reach complete remission with absence of airway obstruction and bronchial hyperresponsiveness. It is uncertain whether this associates with remission of all inflammatory and remodeling asthma features.

Objective: To compare the pathological phenotype of asthmatics with complete and clinical remission and active asthma, with and without the use of inhaled corticosteroids (ICS).

Methods and Measurements: We investigated 165 individuals known with active asthma, upon re-examination having complete remission (CoR, n=18), clinical remission (ClinR, n=44) and current asthma (CuA, n=103, 64 with, 39 without ICS). Inflammatory cells were measured in blood, induced sputum and bronchial biopsies, histamine and ECP in sputum, EPX immunopositivity and remodeling (epithelial changes, E-cadherin expression, basement membrane (BM) thickening, collagen deposition) in bronchial biopsies.

Main Results: Median (range) blood eosinophils from CoR were significantly lower than those from CuA (0.10(0.04-0.24) vs. 0.18(0.02-1.16) $\times 10^9/L$). Bronchial EPX immunopositivity was lower in CoR than in both ClinR and CuA (67(0.5-462) vs. 95(8-5329) and 172(6-5313) pixels). Other inflammatory findings were comparable. BM thickness was lowest in CuA, caused by lower BM thickness in those using ICS (CoR: 6.3(4.7-8.4), ClinR 6.5(3.8-11.7), CuA 5.7(2.8-12.6) and ICS using CuA 5.3(2.8-8.2) μm).

Conclusion: CoR is still accompanied by airway abnormalities since BM thickness is similar in asthmatics with CoR, ClinR and CuA without ICS. Airway eosinophilic activation best differentiates these three groups, signifying their importance in the clinical expression and severity of bronchial hyperresponsiveness in asthma.

INTRODUCTION

Some asthma patients outgrow their disease and reach clinical asthma remission (ClinR), i.e. their asthma symptoms have disappeared even without using asthma medication like inhaled corticosteroids (ICS) and β_2 -agonists. A recent prospective epidemiologic study reported an asthma remission rate of 2 per 100 person years, asthma remission being defined as no asthma symptoms in two consecutive years and no current asthma treatment(1). It is, however, still under debate whether subjects in ClinR have actually outgrown their disease since these subjects may still have (asymptomatic) bronchial hyperresponsiveness (BHR) and/or a low lung function. Indeed, Boulet et al have shown that individuals known with asthma yet without current asthma symptoms or asthma treatment for the past 2 years still had subclinical active disease, characterized by BHR, increased reversibility and low lung function(2). It is well possible that this subclinical active disease is reflecting ongoing airway inflammation and/or remodeling. In line with this, higher eosinophil numbers were reported in bronchoalveolar lavage fluid of atopic children in ClinR (no symptoms for 12 months and no current medication) compared to healthy controls(3).

It has been speculated that the persistence of airway inflammation in ClinR will lead to progressive airway remodeling and consequently an increased risk of eventual asthma relapse(3;4). Indeed, a substantial proportion of subjects in ClinR have a rebound of asthma symptoms in later life(5), suggesting that information provided by the patient on asthma symptoms and medication use is likely insufficient to determine whether asthma is definitively in remission. Therefore, researchers have put forward the term "complete asthma remission (CoR)" signifying the absence of BHR and airway obstruction in addition to the absence of symptoms and asthma treatment(5;6). One might indeed anticipate that the absence of asthma symptoms, BHR and airway obstruction in former asthmatics who currently do not use asthma treatment is accompanied by normal airway wall pathology, i.e. no inflammation or remodeling.

The aim of the present study was to characterize inflammatory phenotypes in a large group of individuals (n=165) with CoR (n=18), ClinR (n=44), and current asthma (CuA, n=103) in airway wall biopsies, induced sputum and blood. Since these phenotypes can be changed or masked by current ICS treatment, we investigated CuA with (n=64) and without (n=39) ICS use in case differences existed between asthma remission and CuA. Thus we investigated the airway pathology of 165 individuals known with active asthma in the past who after re-examination were divided into three groups: CoR, ClinR, and CuA (with and without ICS).

METHODS

SUBJECTS

Subjects with complete asthma remission (CoR, n=18), clinical asthma remission (ClinR, n=44) and current asthma (CuA, n=103) were investigated (Table 1). All had a doctor's diagnosis of asthma, and documented reversibility and BHR to histamine in the past. Subjects were re-examined by lung function, BHR to histamine and/or adenosine 5'-monophosphate (AMP), sputum induction and bronchoscopy with bronchial biopsies. CoR (n=18) was defined as no BHR to histamine (PC20histamine $>32\text{mg/ml}$) and AMP (PC20AMP $>320\text{mg/ml}$), normal lung function (FEV₁ $>90\%$ predicted), absence of asthma medication (ICS or β -agonists) use and absence of asthma symptoms (no wheeze or asthma attacks during the last 3 years)(5;6). ClinR (n=44) was defined as absence of asthma medication (ICS or β -agonists) use and absence of asthma symptoms (no wheeze or asthma attacks during the last 3 years) in former asthmatics despite the presence of BHR to histamine (PC20histamine $>32\text{mg/ml}$) and/or AMP (PC20AMP $>320\text{mg/ml}$). CuA (n=147) was defined as presence of BHR to histamine (PC20histamine $>32\text{mg/ml}$)

and/or AMP (PC20AMP >320mg/ml) and the presence of asthma symptoms (wheeze or asthma attacks during the last 3 years). The CuA group consisted of 64 patients using ICS and 39 not using ICS. The study protocol was approved by the local medical ethics committee. All subjects gave written informed consent.

STUDY DESIGN

All subjects were evaluated with blood sampling, sputum induction and lung function assessment, followed 1 to 2 weeks later by an AMP provocation test. At the third visit, again 1 to 2 weeks later, bronchoscopy with collection of bronchial biopsies was performed. Subjects with a negative AMP provocation test (PC20AMP >320 mg/ml) came for a fourth visit to undergo a histamine provocation test at least 3 weeks later.

MEASUREMENTS

Lung function, sputum induction and bronchoscopy with collection of bronchial biopsies were performed as published previously(7) and are described in detail in the online data supplement. In short, histamine (IBL, Hamburg, Germany) and neutrophil elastase (Hycult, Uden, the Netherlands) in sputum supernatant were determined by ELISA and eosinophilic cationic protein (ECP) by a fluoroenzyme immunoassay (ImmunoCAP ECP, Phadia AB).

Bronchial biopsies were fixed in 4% formalin, processed and embedded in paraffin and cut in 3µm thick sections. Immunohistochemical stainings (except for Ki67 and E-cadherin) were performed using DAKO autostainer (DAKO, Glostrup, Denmark). Immunohistochemistry was performed using antibodies directed against inflammatory cells (see the online data supplement), eosinophilic peroxidase (EPX8), mast cell tryptase (AA1, DAKO), proliferation (Ki67, DAKO), epithelial adhesion (E-cadherin, BD Biosciences, Breda, the Netherlands) and collagen 3 (Southern Biotech, Birmingham, AL, USA). Further details on the used immunohistochemistry and quantification procedures are presented in the online data supplement.

STATISTICS

Analyses were performed using SPSS (version 16.0; SPSS Inc, Chicago, IL, USA). Normality of distributions was assessed by Kolmogorov-Smirnov test. If necessary, normalization by transformation was attempted. Normally distributed data were analyzed with one-way ANOVA and independent sample t-tests, non-normally distributed data with Kruskal-Wallis and Mann-Whitney U tests. Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson tests or Spearman tests (normally and non-normally distributed data (rs), respectively). Normally distributed data, with or without transformation, were analysed by multiple linear regression analysis correcting for sex, age and current smoking. The p-values of the regression analyses are being presented in tables and graphs. Two tailed p-values of <0.05 were considered statistically significant. Further experimental information can be obtained from the online data supplement.

RESULTS

PATIENT CHARACTERISTICS

In total, 165 former asthma patients were investigated: 18 subjects with complete asthma remission (CoR), 44 with clinical asthma remission (ClinR) and 103 current asthmatics (CuA). Table 1 presents their clinical characteristics. Subjects with CoR and subjects in ClinR had a significantly higher FEV1/VC (inspiratory Vital Capacity)

TABLE 1: PATIENT CHARACTERISTICS

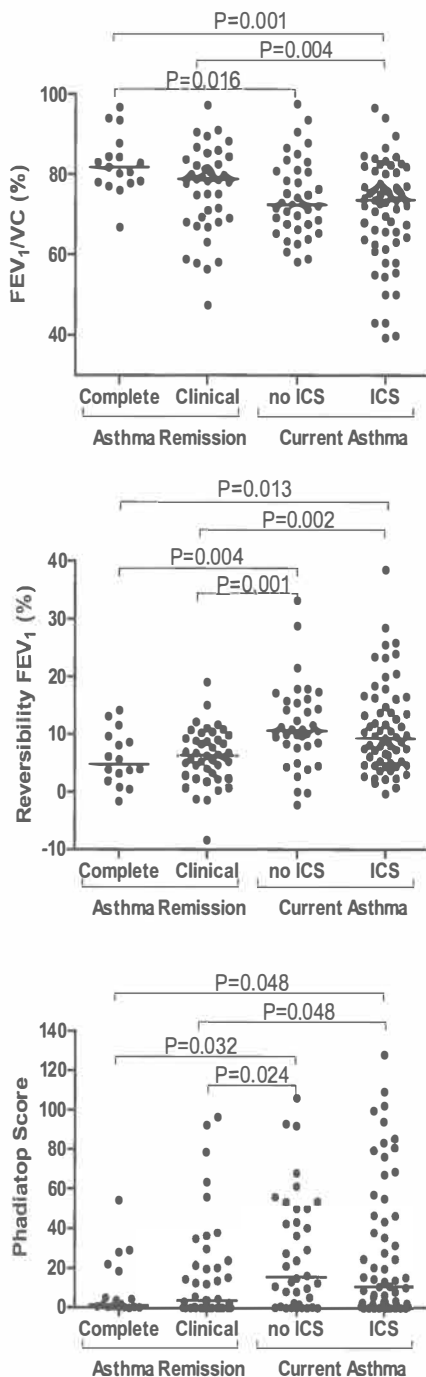
	Complete Asthma Remission	Clinical Asthma Remission	Current Asthma
	N=18	N=44	N=103
PC ₂₀ AMP (mg/ml)	>320	262 (0.02->320) [§]	38.7 (0->320) ^{*,#}
FEV ₁ (%pred)	117 (97-137)	103 (60-123) [§]	101 (42-135) [*]
ICS use (n(%))	-	-	64 (62) ^{*,#}
ICS dose (µg/day)	-	-	800 (28-2000)
Beta-agonist use (n(%))	-	-	80 (78) [#]
Sex (m/f)	7/11	22/22	51/52
Age (year)	48 (17-67)	48 (19-70)	50 (21-71)
FEV ₁ /VC (%)	82 (67-97)	79 (48-97)	73 (39-98) ^{*,#}
Reversibility FEV ₁ (%)	4.8 (-1.7-14.1)	6.2 (-8.4-19.1)	10 (-2.2-38.4) ^{*,#}
Atopy (n (%))	10 (56)	24 (55)	76 (74) [#]
Phadiatop score (quotient)	1.5 (0.2-53.9)	3.9 (0.1-96.5)	13.3 (0.2-128.0) ^{*,#}
Smoking status (n (%))			
current	5 (28)	11 (25)	24 (23)
ex	2 (11)	12 (27)	34 (33)
never	11 (61)	21 (48)	45 (44)
NO alveolar (ppb)	4.3 (0.9-12.3)	4.6 (2.1-12.4)	5.6 (1.5-51.7)
NO bronchial (nL/s)	0.47 (0.09-1.26)	0.57 (0.24-2.72)	0.80 (0.06-10.38)
Blood eosinophils (x10 ⁹ /l)	0.10 (0.04-0.24)	0.16 (0.01-0.50)	0.18 (0.02-1.16) ^{*,#}

Data are presented as median (range), unless stated otherwise. ICS dose is equivalent of beclomethasone. FEV₁ was determined after inhalation of 400 µg of Albuterol; reversibility FEV₁ = change in FEV₁, expressed as increase in percentage predicted normal value after 400 µg of Albuterol. PC₂₀AMP = provocative concentration of adenosine 5' monophosphate causing a 20% fall in FEV₁. Atopy is defined as specific IgE's in patient serum/control serum >1. Phadiatop score = quotient specific IgE in patient serum/control serum. ICS = inhaled corticosteroid, NO = nitric oxide. When applicable corrected for age, sex and smoking using multiple regression analysis.

*: P<0.05, complete asthma remission versus current asthma

#: P<0.05, clinical asthma remission versus current asthma

§: P<0.05, complete asthma remission versus clinical asthma remission



compared to CuA (Table 1, Figure 1A). Reversibility of FEV_1 was significantly lower in both CoR and ClinR compared with CuA (Table 1, Figure 1B). The Phadiatop score, a measure for the severity of atopy, was significantly lower in subjects with both CoR and ClinR in comparison to CuA (Table 1, Figure 1C). Alveolar and bronchial fractions of exhaled breath NO were not significantly different between the three groups (Table 1). Levels of alveolar and bronchial NO fractions correlated with blood eosinophil numbers in the complete population (CoR, ClinR and CuA combined, $rs=0.39$, $p<0.001$ and $rs=0.37$, $p<0.001$, respectively).

EOSINOPHILIC INFLAMMATION IN BLOOD, SPUTUM AND BIOPSIES

The number of blood eosinophils was significantly lower in both CoR and ClinR than in CuA (Table 1, Figure 2A). The percentage of sputum eosinophils and the ECP concentration in sputum supernatant were not significantly different between the three groups (Table 2, Figure 2B,C). However, when the group with CuA was divided in non-ICS users and ICS users, sputum ECP was significantly higher in CuA patients with ICS use than in those with CoR and CuA without ICS use (Figure 2C). Sputum ECP correlated significantly with the percentage of sputum eosinophils in the complete population (CoR, ClinR and CuA combined, $rs=0.37$, $p<0.001$). EPX immunopositivity in the bronchial biopsy tissue was significantly lower in subjects with CoR than in subjects with ClinR and CuA (with and without ICS use, Table 3, Figure 2D).

Figure 1
Patient characteristics. (A) FEV_1/VC (%), (B) Reversibility of FEV_1 (% predicted), and (C) Phadiatop score in subjects with complete asthma remission, clinical asthma remission, no ICS using current asthmatics and ICS using current asthmatics. P-values of FEV_1/VC are after correction for age, sex and smoking using multiple regression analysis. Each circle represents one subject. Horizontal bars represent median values.

Eosinophilic inflammation in blood, sputum and biopsy correlated significantly with each other in the complete population (CoR, ClinR and CuA together), blood eosinophils versus sputum eosinophils (%) $r_s=0.54$, $p<0.001$, blood eosinophils versus EPX immunopositivity $r_s=0.25$, $p=0.005$ and sputum eosinophils (%) versus EPX immunopositivity $r_s=0.39$, $p<0.001$.

OTHER INFLAMMATORY CELLS AND MEDIATORS IN SPUTUM AND BIOPSIES

The percentage of sputum lymphocytes was significantly higher in subjects with CoR than in CuA (Table 2). This was in particular found in CuA with ICS use (1.4 (0-7.3) % in CoR vs. 0.5 (0-6.9) % in CuA without ICS and 0.3 (0-4.1) % in CuA with ICS).

The level of histamine in sputum supernatant was significantly lower in subjects with ClinR compared to CuA (Table 2), and particularly lower than in CuA using ICS (18.5 (0-158.7) ng/mL in ClinR vs. 22.7 (0-139.9) ng/mL in CuA without ICS and 41.7 (4.7-215.0) ng/mL in CuA with ICS).

Besides group differences in eosinophils, we observed no significant differences in any of the other inflammatory cell types studied in bronchial biopsies (Table 3).

TABLE 2: INFLAMMATORY CELLS AND MEDIATORS IN INDUCED SPUTUM

	Complete Asthma Remission N=18	Clinical Asthma Remission N=44	Current Asthma N=103
Sputum not evaluable n (%)	5 (28)	20 (45)	22 (21)
No sputum n (%)	3 (16)	13 (29)	12 (11)
>80% squamous n (%)	1 (6)	4 (9)	7 (7)
<600 cells n (%)	1 (6)	3 (7)	3 (3)
Sputum evaluable n (%)	13 (72)	24 (55)	81 (79)
Total cells ($\times 10^6/l$)	0.31 (0.05-2.90)	0.44 (0.06-2.27)	0.43 (0.03-3.86)
Neutrophils (%)	62 (19-78)	58 (21-94)	57 (16-94)
Macrophages (%)	36 (20-78)	34 (5-77)	36 (5-83)
Eosinophils (%)	0.2 (0-4.6)	0.9 (0-16.8)	0.9 (0-67.1)
Lymphocytes (%)	1.4 (0-7.3)	0.7 (0-5.6)	0.4 (0-6.9)*
Neutrophil elastase ($\mu g/ml$)	1.1 (0-5.0)	1.6 (0.2-5.0)	1.4 (0.2-5.0)
Eosinophilic cationic protein ($\mu g/l$)	17.9 (2.5-172)	24.3 (5.6-2467)	33.6 (4.2-1363)
Histamine (ng/ml)	22.7 (2.8-99.6)	18.5 (0-158.7)	35.4 (0-215.0)#

Data are presented as median (percentage or range). When applicable corrected for age, sex and smoking using multiple regression analysis.

*: $P<0.05$, complete asthma remission versus current asthma

#: $P<0.05$, clinical asthma remission versus current asthma

§: $P<0.05$, complete asthma remission versus clinical asthma remission

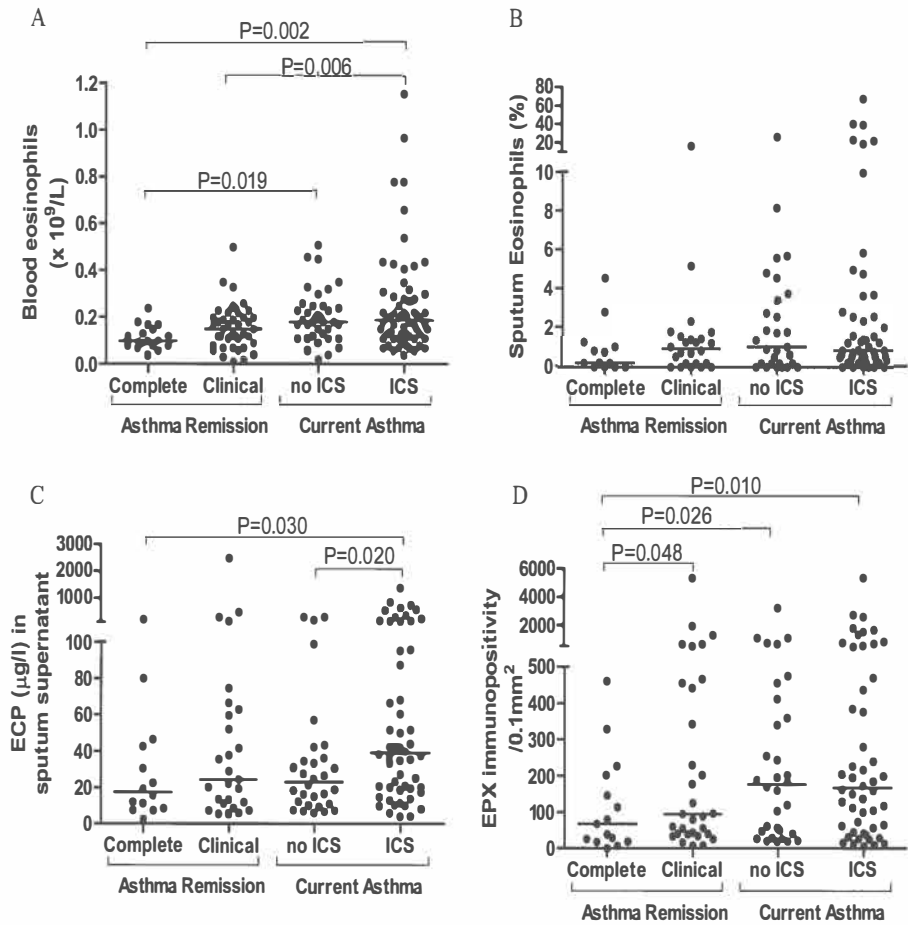


Figure 2
Eosinophilic inflammation. (A) Absolute blood eosinophils ($\times 10^9/L$), (B) Percentage of sputum eosinophils, (C) Eosinophil cationic protein (ECP) in sputum supernatant ($\mu g/L$), and (D) EPX immunopositivity (pixels) in bronchial biopsies in subjects with complete asthma remission, clinical asthma remission, no ICS using current asthmatics and ICS using current asthmatics. P-values of EPX immunopositivity are after correction for age, sex and smoking using multiple regression analysis. Each circle represents one subject. Horizontal bars represent median values.

TABLE 3: INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

	Complete Asthma Remission	Clinical Asthma Remission	Current Asthma
	N=15	N=32	N=82
<i>Submucosal</i>			
CD3 ⁺ T-cells	52 (32-291)	67 (13-294)	68 (4-219)
CD4 ⁺ T-cells	12 (0-168)	21 (0-67)	17 (0-71)
CD8 ⁺ T-cells	28 (7-112)	18 (3-103)	23 (0-205)
CD20 ⁺ B-cells	2 (0-71)	4 (0-98)	3 (0-56)
NP57 ⁺ neutrophils	7 (2-30)	8 (0-46)	6 (0-38)
CD68 ⁺ macrophages	13 (2-86)	16 (0-37)	12 (0-57)
EPX ⁺ eosinophils	1 (0-10)	2 (0-40)	2 (0-32)
AA1 ⁺ mast cells	7 (1-26)	8 (0-20)	8 (0-26)
<i>(De)granulation</i>			
EPX immunopositivity (pixels)	67 (0.5-462)	95 (8-5329) [§]	172 (6-5313) [*]
Degranulated AA1 ⁺ mast cells (%)	71 (20-100)	80 (22-100)	68 (0-100)
<i>Smooth Muscle</i>			
AA1 ⁺ mast cells	1 (0-2)	1 (0-15)	1 (0-6)

Data are presented as median (range). The cell numbers are expressed per 0.1 mm² of tissue. When applicable corrected for age, sex and smoking using multiple regression analysis.

*: P<0.05, complete asthma remission versus current asthma

#: P<0.05, clinical asthma remission versus current asthma

§: P<0.05, complete asthma remission versus clinical asthma remission

AIRWAY WALL REMODELING

Basement membrane (BM) thickness was similar in CoR and ClinR. BM thickness was significantly increased in ClinR compared to CuA (Table 4, Figure 3). When ICS use was taken into account, BM thickness was significantly higher in both ClinR and CuA without ICS use than in CuA with ICS use (Figure 3).

No significant differences were observed between the three groups in any of the other investigated remodeling parameters (Table 4).

TABLE 4: REMODELING IN BRONCHIAL BIOPSIES

	Complete Asthma Remission	Clinical Asthma Remission	Current Asthma
	N=15	N=32	N=82
<i>Bronchial epithelium</i>			
Goblet cells (cells/mm BM)	34 (0-73)	32 (2-220)	36 (5-119)
Normal Epithelium (%)	6.8 (0-32.2)	8.9 (0-29.1)	9.0 (0-65.3)
Metaplasia (%)	0 (0-21.2)	0 (0-26.2)	0 (0-19.4)
Ki67 ⁺ intact epithelium (%)	2.9 (0-43.1)	2.8 (0-13.6)	3.7 (0-24.2)
Ki67 ⁺ basal epithelium (%)	2.7 (0.2-19.2)	3.1 (0.1-15.5)	3.1 (0-28.9)
E-cadherin ⁺ intact epithelium (%)	100 (0-100)	100 (0-100)	86.4 (0-100)
<i>Basement membrane</i>			
Thickness (μm)	6.3 (4.7-8.4)	6.5 (3.8-11.7)	5.7 (2.8-12.6) [#]
<i>Submucosa</i>			
Collagen 3 (% stained tissue)	38.8 (14.5-54.9)	36.7 (8.2-64.5)	41.9 (13.9-77.9)

Data are presented as median (range). When applicable corrected for age, sex and smoking using multiple regression analysis.

*: P<0.05, complete asthma remission versus current asthma

#: P<0.05, clinical asthma remission versus current asthma

§: P<0.05, complete asthma remission versus clinical asthma remission

Figure 3

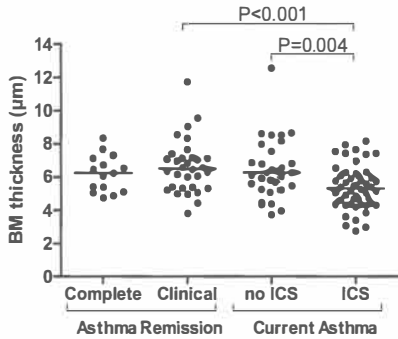


Figure 3.

Airway remodeling. (A) Basement membrane thickness in subjects with complete asthma remission, clinical asthma remission, no ICS using current asthmatics and ICS using current asthmatics. P-values are after correction for age, sex and smoking using multiple regression analysis. Each circle represents one subject. Horizontal bars represent median values.

DISCUSSION

It has been long debated whether asthma can go in true remission. Our study shows that asthma can go in remission when considering the clinical characteristics of asthma, i.e. those in remission had no asthma symptoms, no bronchial hyperresponsiveness (BHR) and had normal lung function despite not using asthma treatment for at least three years. We demonstrate, however, that airway remodeling persists, as evidenced by a similar basement membrane thickness in those with complete asthma remission (CoR), clinical asthma remission (ClinR) and in current asthmatics (CuA) without ICS use. Of particular interest is the observation that eosinophilic inflammation was best differentiating CoR from CuA. Moreover eosinophilic activation in the airway wall, as indicated by increased degranulation in the EPX staining was significantly lower in subjects with CoR than those with ClinR and with CuA.

It has been previously shown that subjects with ClinR (no asthma symptoms and medication for 12 months) had ongoing airway inflammation and remodeling as reflected by increased numbers of eosinophils, T cells and mast cells in the airway mucosa and thickening of the reticular basement membrane when compared to control subjects without asthma(4). However, these individuals still had BHR and it is evident that persistence of BHR is abnormal. Also, asymptomatic BHR is a risk factor for future development of asthma symptoms in epidemiological studies(9). Thus, our study supports our previous findings(6;10) that it is indeed possible that asthma patients who have expressed BHR accompanied by asthma signs and symptoms are capable to lose these clinical hallmarks of asthma. We have previously published in a different group of asthmatics that basement membrane thickness was significantly larger in asthmatics than in age- and smoking matched healthy individuals(11). Thus, the current study for the first time suggests that, even with complete asthma remission, some remodeling remains in the airways, likely as a sign of past disease or scarring.

There are some limitations to our study. We investigated asthmatics at middle age and remission may have different underlying pathology in childhood remission. It was not possible to assess the duration of asthma in our study population, since at initial testing, when all participants had active asthma, it was not asked at which age asthma started. Therefore we cannot exclude that duration of asthma may have contributed to our findings as well. Nevertheless, we have investigated a considerable number of adult asthmatics with complete remission and our findings suggest that at this age remodeling may still be present. Finally, we found that the percentage of sputum lymphocytes was significantly higher in subjects with CoR than in CuA, particularly in CuA with ICS use. Since we did not perform subset analysis of these lymphocytes further interpretation of this part of the results is not possible.

Asthmatics with remission, be it CoR or ClinR, were less frequently atopic and had significantly lower Phadiatop scores, implying less severe atopy than those with CuA. This was paralleled by differences in eosinophilic activation in airway wall biopsies and may well reflect that asthmatics in remission are less hampered by allergic stimuli as present in the environment. It remains to be established whether the lower atopic prevalence in those with remission is due to loss of allergies during ageing, as has been shown previously(5). However, this will not likely affect results of our study since all groups had a comparable age of around 50 years when investigated. It is of interest that sputum eosinophil percentages were not significantly different in subjects with and without remission, suggesting that it is not merely the presence of eosinophils, but their activation state in the airway wall, as reflected by EPX staining, that drives their significant role in active disease. Sputum may thus constitute a waste basket of the presence of eosinophils in the airway wall, where it is the activation of these eosinophils that contributes to active asthma.

It was an unexpected finding that symptomatic CuA patients using ICS had more

severe eosinophilic inflammation than those not using ICS, as shown by higher blood eosinophil numbers and sputum ECP. This may reflect that asthma was not under control in CuA patients using ICS, and higher doses may be necessary to reach control(12). As already mentioned, BM thickness was lower in CuA patients using ICS, showing that asthma symptoms are not driven by this remodeling feature.

Functional abnormalities in asthma such as BHR are thought to be caused by airway inflammation and/or structural changes of the airways, like smooth muscle hyperplasia, airway wall thickening and edema. These structural changes, known as airway remodeling, are thought to appear early in the onset of the disease, sometimes even before symptoms appear(13). The exact physiological consequences of airway wall remodeling are not understood. We here show that airway remodeling is also present in subjects in CoR. This suggests that BM thickening per se is not responsible for the presence of BHR. In contrast, activation of eosinophils in the airway wall is almost absent in CoR and present in those with CuA, suggesting that this may contribute to the presence and/or severity of BHR. Nevertheless, other factors have to drive the presence and severity of BHR as well, since eosinophilia was absent in a proportion of our CuA patients.

The question arises if airway remodeling is an inherent feature of asthma, i.e. if it remains even though at extensive clinical examination asthma is in CoR. If this is true, then one might interpret the presence of increased BM thickening as a pending risk of asthma relapse. Alternatively, this merely reflects an end-stage disease with some "scarring" as a sign that an individual has had asthma. There are indications that remodeling is a process that is amenable to treatment. Indeed two studies have shown that prolonged treatment with ICS can reduce BM thickness(14;15). Moreover, collagen deposition(16) and airway vascularity(17) can improve with ICS use and are therefore also reversible. In the light of these observations it is of interest that CuA patients using ICS in our study had lower BM thickness than those without ICS. This may thus simply result from ICS treatment for a prolonged time. However, despite this, patients still remain symptomatic. Therefore, we conclude that BM thickness itself does not associate with the clinical expression of asthma, but that other factors imprint on BM thickness that affect the severity of the disease and treatment requirements.

Epithelial cells are the first barrier against inhaled allergens, hence these cells may be important for the induction, maintenance and severity of asthma. We have previously shown that smoking asthmatics have more respiratory symptoms and epithelial damage than non-smoking asthmatics(7). We had anticipated that CuA patients would have more epithelial changes than those with ClinR and CoR. However, the percentage intact epithelium and its expression of E-cadherin, an adhesion protein, were similar between the groups. With respect to epithelial damage, we conclude that this is not differentiating remission from CuA. This does, of course not preclude that the activation status of epithelial cells is different. It may well be that the production of chemo-attractive cytokines by epithelium is increased in CuA compared to remission, which then adds to attracting eosinophils to the airway wall as observed in our study.

In summary, we studied a group of 164 adult individuals known with active asthma in the past, including individuals with CoR, i.e. those being asymptomatic without airway obstruction, BHR and any respiratory treatment, individuals with ClinR (i.e. asymptomatic and without treatment) and with CuA. The important message of this paper is that asthma can go in full remission according to all clinical phenotypes that characterize asthma, but that BM thickening, which accompanies asthma, persists. A second message of the paper is that airway wall eosinophilic activation differentiates CoR from ClinR and CuA. This signifies the importance of eosinophil activation for the clinical expression of asthma and the severity of BHR.

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Persisting remodeling and less airway wall eosinophil activation in complete remission of asthma

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Online data supplement

EXCLUSION CRITERIA

Main exclusion criteria were: FEV₁ <1.2 L, bronchiectasis, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within the last 2 months before inclusion.

LUNG FUNCTION

Lung function tests were performed with a daily calibrated spirometer according to standardized guidelines as described previously(1). FEV₁ was measured with a calibrated water-sealed spirometer according to standardized guidelines(1;2). Reversibility of FEV₁ (% predicted) was measured after administration of 400µg Albuterol. BHR to AMP was tested with a method adapted from Cockcroft and coworkers(3) using, after an initial nebulized 0.9% saline challenge, 2-min tidal breathing at 5-min intervals and doubling concentrations of AMP ranging from 0.04 to 320 mg/ml. BHR to histamine was tested as reported previously(4), using 30-second tidal breathing and doubling concentrations of histamine ranging from 0.13 to 32 mg/ml.

ALVEOLAR AND BRONCHIAL NITRIC OXIDE

Exhaled nitric oxide (NO) was measured on the Aerocrine NO system (Niox, Aerocrine AB, Stockholm, Sweden) in accordance with international guidelines(5). Alveolar NO concentrations (ppb) and bronchial NO fluxes (nL/s) were assessed according to Tsoukias and George(6) with some modifications(7).

ATOPY

Atopy was determined by the Phadiatop screening test(8) with the ImmunoCap system (Phadia AB, Uppsala, Sweden). The Phadiatop score is expressed as quotient, i.e. fluorescence of the serum of interest divided by the fluorescence of a control serum. Positive Phadiatop, and thereby atopy, was defined as serum/control serum >1.

INDUCED SPUTUM

Sputum was induced by 5% hypertonic saline aerosols over 3 consecutive periods of 5 min. Cytospins of processed whole sputum samples were stained with May- Grünwald-Giemsa and cell differentials were determined by a blinded observer by counting a total of 600 viable, non-squamous cells. Sputum was not used if percentage of squamous cells was greater than 80% or when the total number of non-squamous cells exceeded 600. Histamine (IBL, Hamburg, Germany) and neutrophil elastase (Hycult, Uden, the Netherlands) in sputum supernatant were determined by ELISA and eosinophilic cationic protein (ECP) by a fluoroenzyme immunoassay (ImmunoCAP ECP, Phadia AB).

BRONCHIAL BIOPSIES

After local anaesthesia, bronchial biopsies were obtained using a flexible bronchoscope (Olympus BF P20 or BF XT20) from segmental divisions of the main bronchi. Biopsies were fixed in 4% formalin, processed and embedded in paraffin and cut in 3µm thick sections. Morphological features were determined on sections stained with hematoxylin and eosin (HE) and with periodic acid Schiff. Immunohistochemical stainings (except for Ki67 and E-cadherin) were performed using the DAKO autostainer (DAKO, Glostrup, Denmark). The slides were included in a random fashion in each run to avoid groupwise staining. Immunohistochemistry was performed using antibodies directed against T-cells: (CD3, DAKO; CD4, Novocastra, Newcastle, UK; and CD8, DAKO), B-cells (CD20, DAKO), neutrophil elastase (NP57, DAKO), macrophages (CD68, DAKO), eosinophilic peroxidase (EPX(9)), mast cell tryptase (AA1, DAKO), proliferation (Ki67, DAKO), epithelial adhesion (E-cadherin, BD Biosciences, Breda, the Netherlands) and collagen 3 (Southern Biotech, Birmingham, AL, USA). In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) and the chromogen NovaRED (Vector Labs, Burlingame, USA). EPX was detected via biotinylated anti-mouse IgG1

(Southern Biotech), alkaline phosphatase-labeled conjugate (DAKO) and permanent Red (DAKO). Ki67, E-cadherin and collagen 3 were detected with two peroxidase-labelled conjugates (both DAKO) and 3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands).

All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). Quantification was performed on the largest of three biopsy sections. Inflammatory cell numbers were quantified by counting the number of positively stained cells in the submucosal area 100µm under the basement membrane (BM), in a total area of 0.1mm² per biopsy sample. Activation of eosinophils and mast cells was determined by their degree of degranulation. Degranulation of eosinophils was determined by computer-assisted quantification of EPX immunopositivity in the submucosa. The percentage of degranulated AA1+ cells was determined by counting intact (dense, compact AA1+ cells with unbroken boundaries) and degranulated mast cells (all other AA1+ cells), as described previously(10). Goblet cell numbers were counted on PAS-stained biopsy sections and expressed per mm of BM. Epithelial layer integrity was assessed on HE-stained biopsy sections and expressed as the percentage of BM covered with 1) normal, intact epithelium (basal and ciliated columnar epithelial cells) and 2) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). Epithelial proliferation was determined by counting the number of Ki67+ cells in intact epithelium and basal epithelium. Epithelial adhesion was determined by assessment of the percentage of BM covered with E-cadherin+ intact epithelium. BM thickness was calculated based on computer-assisted measurements of BM surface area and BM length. Submucosal deposition of collagen 3 was determined by computer-assisted image analysis in an area of 200µm under the BM.

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AMP Responsive Asthma is Characterized by Eosinophils and Basement Membrane Thickening

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ABSTRACT

Rationale: Asthma is characterized by hyperresponsiveness, inflammation and remodeling. We have shown that responsiveness to adenosine 5'monophoshate (AMP) constitutes an indirect determinant of airway inflammation, assessed in induced sputum. Not all asthmatics express an AMP-responsive phenotype.

Objective: To assess whether an AMP-responsive phenotype in asthma associates with pathological changes, focussing on airway inflammation, remodeling and adenosine receptor expression.

Methods: 37 mild, steroid-naïve, non-smoking asthma patients with (n=20) and without (n=17) AMP hyperresponsiveness were investigated.

Measurements: Inflammatory cell numbers, remodeling parameters and adenosine receptor expression were assessed in induced sputum and bronchial biopsies.

Main Results: AMP-responsive asthmatics were more frequently atopic and had higher airway wall eosinophil numbers and eosinophil degranulation. More severe AMP responsiveness associated with a thicker basement membrane which, in turn, correlated with higher eosinophil numbers. Adenosine receptor expression in bronchial biopsies and the number of mast cells expressing A2a or A2b receptors were comparable in asthmatics with and without an AMP-responsive phenotype.

Conclusion: AMP-responsive asthma has a distinct pathological phenotype, characterized by increased eosinophilic inflammation and basement membrane thickness. As eosinophilic inflammation mostly responds well to corticosteroids, the AMP test might be of use to identify corticosteroid responsiveness in asthma.

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways characterized by variable airway obstruction and bronchial hyperresponsiveness (BHR). The presence and severity of BHR is generally assessed by provocation with histamine or methacholine, both acting mainly by direct stimulation of airway smooth muscle cells. BHR is therefore dependent on smooth muscle contraction, and additionally on the presence and severity of airway inflammation and airway remodeling. BHR can also be assessed by inhaling stimuli that indirectly cause smooth muscle contraction, e.g. by inflammatory cell activation. Adenosine 5'-monophosphate (AMP) is such a stimulus, which upon inhalation is rapidly converted to adenosine. Adenosine targets cells by interaction with specific receptors classified as the A₁, A_{2a}, A_{2b} and A₃ receptors(1). While these receptors are present on a wide variety of cell types, inhalation of adenosine is thought to lead to bronchoconstriction mainly after binding of A_{2b} receptors on mast cells, resulting in mast cell degranulation(2;3). This suggests that AMP responsiveness is dependent on airway inflammation. This is supported by our previous observation that PC₂₀AMP (provocative concentration of AMP causing a 20% fall in FEV₁) is more closely associated with eosinophilic inflammation in sputum than PC₂₀methacholine(4). Moreover, steroid treatment improves PC₂₀AMP more rapidly than PC₂₀methacholine, an improvement solely related to a reduction in sputum inflammation(5). Based on these findings, one can anticipate that AMP provocation will constitute a good clinical test to monitor eosinophilic inflammation and treatment effects in asthma albeit that the above mentioned studies were performed in induced sputum, which is only a surrogate marker for airway inflammation. Until now, no data on AMP hyperresponsiveness and airway inflammation in lung tissue or biopsies are available.

Not all asthma patients exhibit AMP hyperresponsiveness and the reported prevalence of a positive PC₂₀AMP varies from 50 to 89%(4;6). This would be a drawback to the clinical use of PC₂₀AMP. Therefore, it is of interest to characterize the AMP-responsive phenotype by investigating the pathology of asthmatics with and without a positive or negative response to AMP provocation.

Therefore, we investigated sputum as well as airway biopsies for pathological differences between AMP-responsive and unresponsive asthmatics with respect to inflammation and remodeling. Additionally, we studied adenosine receptor expression in the airway wall and on mast cells in these patients to unravel further mechanisms of AMP responsiveness.

METHODS

SUBJECTS

Non-smoking asthma patients (n=37) aged between 35 and 70 years participated. All patients originated from cohorts investigated earlier by our research group and all had a doctor's diagnosis of asthma and a documented PC₂₀histamine \leq 32 mg/ml in the past(7). AMP responsiveness was assessed and two groups were identified: 1) negative PC₂₀AMP (PC₂₀AMP >320 mg/ml and PC₂₀histamine <32 mg/ml) and 2) positive PC₂₀AMP (PC₂₀AMP \leq 320 mg/ml). Main exclusion criteria were: FEV₁ <1.2 L, bronchiectases, upper respiratory tract infection (e.g. colds) and/or use of antibiotics the last 2 months before inclusion, the use of corticosteroids the last 5 years before inclusion or more than 5 years of cumulative corticosteroid use in the distant past. The study protocol was approved by the local medical ethics committee. All subjects gave their written informed consent.

STUDY DESIGN

Induced sputum samples, blood samples and lung function (flow-volume, CO diffusion) were obtained. An AMP provocation test was performed, followed 1-2 weeks later by a bronchoscopy with bronchial biopsies. Subjects with a negative AMP provocation test (PC₂₀ AMP >320 mg/ml) were subjected to a histamine provocation test >3 weeks later to confirm the presence of BHR.

LUNG FUNCTION

FEV₁ was measured with a calibrated water-sealed spirometer (Lode Spirograph D53, Lode Instruments, Groningen, the Netherlands). Reversibility of FEV₁ (% predicted) was measured after administration of 400 µg of Albuterol and expressed as percentage. Provocation tests were performed with a method adapted from Cockcroft and coworkers(8). After an initial nebulized 0.9% saline challenge, subjects inhaled doubling concentrations of AMP (0.04 to 320 mg/ml) by 2-min tidal breathing, at 5-min intervals. BHR to histamine was tested as reported previously(9), using 30-second tidal breathing and doubling concentrations ranging from 0.13 to 32 mg/ml. To enable better estimation of the airway reactivity in asthmatics with a negative PC₂₀AMP, we also analyzed the AMP challenges as the AMP dose-response slope, calculated by dividing the maximum fall in FEV₁ (l) by the dose of AMP at reaching the threshold (mg)(10).

ATOPY

Total serum IgE (IU/L) was measured by a solid-phase immunoassay (VIDAS total IgE kit, BioMérieux, Marcy l'Etoile, France). Atopy was determined by the Phadiatop screening test with the ImmunoCap system (Phadia AB, Sweden), results being presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Positive Phadiatop was defined as patient serum/control serum >1.

SPUTUM INDUCTION, PROCESSING AND MEASUREMENTS

Sputum was induced by inhalation of 5% hypertonic saline aerosols over 3 consecutive periods of 5 min. Processed whole sputum samples were stained with May-Grünwald-Giemsa (MGG) to obtain cell differentials and counting 600 viable, non-squamous cells. Sputum was not used if the percentage squamous cells was >80% or the total number of non-squamous cells was <600. Secreted mucin was measured by double-sandwich ELISA (Covance, Emeryville, USA), with the 17B1 protein-G purified antibody and 17Q2 alkaline phosphatase-conjugated antibody specifically binding to the granules of the surface goblet cells and mucous gland cells, as previously described(11).

Collection, processing and immunohistochemical staining of bronchial biopsies

After local anaesthesia, bronchial biopsies were obtained using a flexible bronchoscope (type Olympus BF P20 or BF XT20) from segmental divisions of the main bronchi. The biopsies were fixed in 4% buffered formalin, processed and embedded in paraffin. Bronchial biopsies were cut in 3 µm thick sections.

All histochemical stainings (PAS and HE) were performed at one day. All immunohistochemical stainings (except for Ki67 and E-cadherin) were performed in an automated system using the DAKO autostainer in three consecutive runs per cell marker. The slides were included in random fashion in each run, to avoid groupwise staining.

Immunohistochemistry was performed on biopsies using antibodies directed against T-cells (CD3, DAKO, Glostrup, Denmark, CD4, Novocastra, Newcastle, UK, CD8, DAKO), B-cells (CD20, DAKO), neutrophil elastase (NP57, DAKO), macrophages (CD68, DAKO), eosinophilic peroxidase (EPX (12)), mast cell tryptase (AA1, DAKO), proliferation (Ki67, DAKO), epithelial adhesion (E-cadherin, BD Biosciences, Breda, the Netherlands), collagen 3 (Southern Biotechnology, Birmingham, AL, USA), decorin (dermatan sulfate proteoglycan, Seikagaku, Tokyo, Japan), versican (large proteoglycan (versican), Seikagaku) and adenosine receptors A2a, A2b (both from Alpha Diagnostics, San Antonio, TX, USA) and A3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Stainings were performed as described previously(13) and details about the procedure can be obtained from the online supplement.

QUANTIFICATION OF INFLAMMATORY CELL NUMBERS

Quantification of inflammatory cell numbers and activation was performed by a blinded observer using computerized-assisted image analysis at a magnification of

200x (Qwin, Leica Microsystems). The number of positively stained inflammatory cells was counted in the submucosal area 100µm under the basement membrane (BM), in a total area of 0.1mm² per biopsy sample. Additionally, mast cells were quantified in the smooth muscle area, which could be readily identified by its morphologic appearance. A minimal area of 0.05 mm² airway smooth muscle was considered sufficient for the assessment of mast cell infiltration.

QUANTIFICATION OF ACTIVATED EOSINOPHILS AND MAST CELLS

Activation of eosinophils and mast cells was determined by their degree of degranulation. EPX staining showed a widely spread distribution of eosinophilic granules, not necessarily in close proximity of EPX+ cells. Therefore, degranulation of eosinophils was determined by quantification of the EPX immunopositive area by computerized-assisted image analysis. AA1+ mast cell granules were only observed in close proximity of AA1+ cells and therefore, the percentage of degranulated AA1+ cells was determined by counting(14). In short, positively stained nucleated mast cells were classified as intact if they were dense, compact, had unbroken boundaries and did not have any surrounding positively stained granules. All other nucleated AA1+ cells were classified as degranulated.

QUANTIFICATION OF REMODELING

The number of goblet cells was counted on PAS-stained biopsy sections and expressed per mm of BM. Epithelial integrity was determined using HE-stained biopsy sections and was expressed as the percentage of BM that was covered with either intact epithelium (a layer of basal and ciliated columnar epithelial cells without detachment from the BM), only basal epithelial cells, metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells) or the percentage of BM that was denuded from epithelial cells. Epithelial proliferation was determined by counting the number of Ki67-positive epithelial nuclei in intact and basal epithelium. Epithelial adhesion was determined by expression of the adhesion marker E-cadherin. In intact epithelium and basal epithelium the percentage of the BM that was covered with either E-cadherin-positive or negative epithelium was determined. BM thickness was calculated by dividing the BM surface area by the BM length(15). Expression of collagen 3, versican, and decorin was measured in the submucosal area 200µm under the BM by computerized-assisted image analysis, and was expressed as the percentage of positive tissue per biopsy section.

QUANTIFICATION OF ADENOSINE RECEPTOR EXPRESSION

A2a, A2b and A3 receptor expression was determined quantitatively using three categories as described previously(16). In the epithelium and smooth muscle, expression was categorized as 0 (negative), 1 (positive) or 2 (strongly positive) and expression in submucosal inflammatory cells and vascular endothelium was categorized as 0 (no positive cells/vessels), 1 (few positive cells/vessels) or 2 (many positive cells/vessels). In the submucosal area (excluding the epithelial layer) and in the smooth muscle we counted the number of AA1+/A2a receptor and AA1+/A2b receptor double-positive cells and expressed them as the percentage of the total number of AA1+ mast cells.

STATISTICS

All analyses were performed using SPSS (version 16.0; SPSS Inc, Chicago, IL, USA). Normality of distributions was assessed using the Kolmogorov-Smirnov test. When necessary, normalization by transformation was attempted. To test for differences between the negative PC20AMP and positive PC20AMP groups independent samples T-tests were used for normally distributed data and Mann-Whitney U tests for non-normally distributed data. Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson (for normally distributed data; expressed as r) or Spearman (for non-normally distributed data; expressed as rs) tests. Two tailed p-values of <0.05 were considered statistically

significant.

RESULTS

PATIENT CHARACTERISTICS

Thirty-seven asthmatic patients were included, 20 patients with and 17 patients without a positive PC₂₀AMP (Table 1). The latter group did demonstrate responsiveness to histamine. Lung function parameters, i.e. FEV₁ (%pred), FEV₁/VC and reversibility were all comparable between asthmatics with a positive and negative PC₂₀AMP. Asthmatics with a positive PC₂₀AMP were more often atopic and had a significantly higher Phadiatop score than asthmatics with a negative PC₂₀AMP. The Phadiatop score positively correlated with the severity of AMP responsiveness (expressed as the AMP dose-response slope, Spearman correlation $rs=0.50$, $p=0.002$). Blood eosinophil numbers were not significantly different in asthmatics with a positive and a negative PC₂₀AMP, but they correlated significantly with AMP dose-response slope in the whole group of 37 asthmatics ($rs=0.39$, $p=0.017$, Figure 1A-B).

EOSINOPHILS AND MAST CELLS IN BRONCHIAL BIOPSIES

Bronchial biopsies were successfully obtained from 29 of the 37 asthma patients. Asthmatics with a positive PC₂₀AMP had higher eosinophilic inflammation as shown by a higher number of submucosal EPX+ eosinophils and higher EPX immunopositivity (measure of eosinophilic degranulation products) than those with a negative PC₂₀AMP (Figure 1C, E-F, Table 2). Bronchial eosinophils, both EPX+ eosinophils and EPX immunopositivity, correlated positively with AMP dose-response slope ($rs=0.59$, $p=0.001$ and $rs=0.64$, $p<0.001$, respectively (Figure 1D)).

TABLE 1: PATIENT CHARACTERISTICS

	Negative PC ₂₀ AMP (n=17)	Positive PC ₂₀ AMP (n=20)
PC ₂₀ AMP (mg/ml)	>320	18.2 (0.02-174.3)
AMP dose-response slope	0.4 (-0.3-1.8)	27.5 (1.9-30150.0)*
Sex (M/F)	9/8	14/6
Age (years)	48 (35-70)	53 (38-62)
FEV ₁ (% predicted)	100 (76.9-125.0)	99 (84.8-130.9)
FEV ₁ /VC (%)	78.2 (58.0-90.7)	72.0 (56.4-83.8)
Reversibility FEV ₁ (%)	6.5 (-1.4-15.0)	8.5 (-8.4-28.7)
Atopic (n (%))	9 (53%)	17 (85%)*
Phadiatop score (quotient)	2.6 (0.1-29.5)	20.0 (0.3-92.1)*
IgE (IU/L)	55 (13-246)	81 (11-558)

Data are presented as median (range), unless stated otherwise. PC₂₀AMP = provocative concentration of adenosine 5' monophosphate causing a 20% fall in FEV₁. The AMP dose-response slope was calculated by dividing the maximum fall in FEV₁ (l) by the dose of AMP at reaching the threshold (mg). FEV₁ (% predicted) was determined after inhalation of 400 µg of Albuterol; reversibility FEV₁ = change in FEV₁, expressed as increase in percentage predicted normal value after 400 µg of Albuterol; Atopy is defined as specific IgE's in patient serum/control serum >1; n=number. Phadiatop score = quotient specific IgE in patient serum/control serum.

*: P<0.05

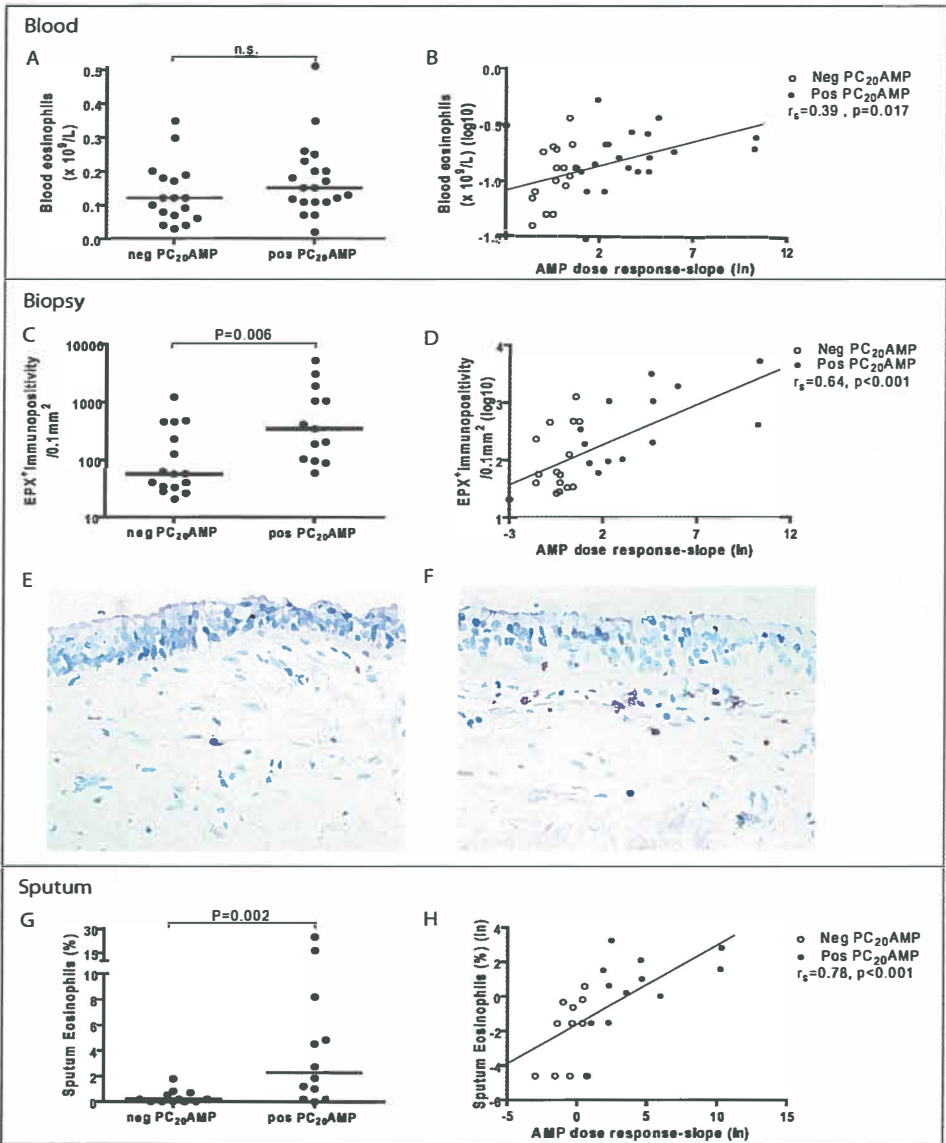


Figure 1
Eosinophilic inflammation and AMP responsiveness. (A) Blood eosinophils in asthmatics with a negative or a positive PC₂₀AMP. (B) Correlation between blood eosinophils (log10 transformed) and AMP dose-response slope (ln transformed). (C) Degranulation of eosinophils, measured by EPX immunopositivity, in bronchial biopsies of asthmatics with a negative or a positive PC₂₀AMP. (D) Correlation between EPX immunopositivity (log10 transformed) and AMP dose-response slope (ln transformed). (E-F) Representative pictures of EPX staining (pink) in asthmatic patients, assessing both a negative (E) and positive (F) PC₂₀AMP patient. Notice the extracellular EPX⁺ granules observed in the PC₂₀AMP patient in addition to the intracellular EPX⁺ granules, quantified as EPX immunopositivity. Original magnification 400x. (G) Sputum eosinophil percentages in asthmatics with a negative or a positive PC₂₀AMP. (H) Correlation between sputum eosinophil percentages (ln transformed) and AMP dose-response slope (ln transformed). Each closed circle represents one subject, open circles are asthmatics with a negative PC₂₀AMP and closed circles are asthmatics with a positive PC₂₀AMP. Horizontal bars represent median values.

In the submucosa, both the number of mast cells and the percentage of degranulated mast cells were comparable between asthmatics with a positive and negative PC₂₀AMP (Table 2). In the airway smooth muscle, the number of mast cells did not differ significantly between the two groups (Table 2). The number of airway wall T-cells, B-cells, neutrophils and macrophages was not significantly different between the two groups (Table 2).

REMODELING IN BRONCHIAL BIOPSIES

Basement membrane thickness derived from H&E stained lung biopsy sections (Figure 2A) was significantly higher in bronchial biopsies of asthmatics with than without a positive PC₂₀AMP (Figure 2B). Basement membrane thickness correlated strongly with the AMP dose-response slope (Figure 2C). Moreover, basement membrane thickness correlated significantly with eosinophilic inflammation, i.e. the percentage of sputum eosinophils ($r_s=0.67$, $p=0.001$) and the number of EPX⁺ eosinophils ($r_s=0.51$, $p=0.006$) and EPX immunopositivity ($r_s=0.54$, $p=0.003$) in the airway wall.

TABLE 2: INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

	Negative PC ₂₀ AMP (n=16)	Positive PC ₂₀ AMP (n=13)
<i>Submucosal</i>		
CD3 ⁺ T-cells	58 (33-246)	85 (21-136)
CD4 ⁺ T-cells	20 (9-67)	26 (2-57)
CD8 ⁺ T-cells	18 (3-112)	19 (1-84)
CD20 ⁺ B-cells	4 (0-46)	3 (0-22)
NP57 ⁺ neutrophils	10 (3-30)	5 (1-46)
CD68 ⁺ macrophages	16 (3-37)	16 (4-30)
EPX ⁺ eosinophils	2 (0-26)	6 (2-40)*
AA1 ⁺ mast cells	3 (0-19)	6 (0-13)
<i>(De)granulation</i>		
EPX immunopositivity (pixels)	56 (21-1268)	343 (60-5329)*
Degranulated AA1 ⁺ mast cells (%)	68.3 (33-100)	75 (50-100)
<i>Smooth Muscle</i>		
AA1 ⁺ mast cells	0.5 (0.2-15.3)	1.2 (0-6.0)

Data are presented as median (range). The cell numbers are expressed per 0.1 mm² of tissue.

*: $P<0.05$

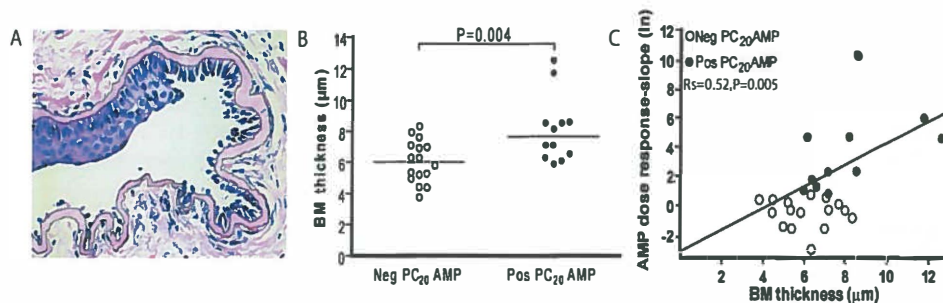


Figure 2

Basement membrane thickness and AMP responsiveness. (A) Representative photomicrograph of an H&E stained section typical of the photographs used to measure basement membrane (BM) thickness. The black line indicates the measured area. Original magnification 400x. (B) BM thickness in asthmatics with a negative (open circles) or a positive (closed circles) PC20AMP. (C) Correlation between BM thickness and the AMP dose-response slope (ln transformed). Each circle represents one subject. Horizontal bars represent median values.

Sputum of asthmatics with a positive PC20AMP contained more mucin protein than in those with a negative PC20AMP (Table 3). The airway epithelium of asthmatics with a positive PC20AMP contained a higher percentage of metaplastic epithelium than asthmatics with a negative PC20AMP (Table 3). Sputum mucin protein and metaplastic epithelium did not significantly correlate with the AMP dose-response slope. Other features of epithelial integrity including the presence of intact, basal or denuded epithelium, the number of goblet cells, epithelial proliferation (Ki67 expression), and the epithelial adhesion (E-cadherin expression) were all comparable in both groups (Table 3). Moreover, no significant differences were observed in the submucosal expression of extracellular matrix components (i.e. collagen 3, versican and decorin) between the asthmatics with a positive and a negative PC20AMP (Table 3).

TABLE 3: REMODELING IN SPUTUM AND BIOPSIES

	Negative PC ₂₀ AMP (n=16)	Positive PC ₂₀ AMP (n=13)
<i>Sputum</i>		
Mucin in sputum (units)	0.4 (0.01-1.6)	0.75 (0.2-4.0)*
<i>Bronchial epithelium</i>		
Goblet cells (cells/mm BM)	29 (4-78)	36 (7-220)
Intact Epithelium (%)	8.9 (0-34.2)	4.2 (0-29.6)
Basal Epithelium (%)	57.8 (13.5-100)	58.7 (20.6-92.9)
Denuded Basement Membrane (%)	14.4 (0-58.8)	9.2 (0-34.3)
Metaplasia (%)	0 (0-5.6)	0 (0-26.2)*
Ki67 ⁺ intact epithelium (%)	1.5 (0-6.3)	1.4 (0-24.2)
Ki67 ⁺ basal epithelium (%)	1.8 (0.3-9.0)	1.3 (0-5.5)
E-cadherin ⁺ intact epithelium (%)	65.7 (0-100)	58.3 (0-100)
E-cadherin ⁺ basal epithelium (%)	94 (67-100)	96.6 (10-100)
<i>Submucosa</i>		
Collagen 3 (% stained tissue)	36.3 (8.6-64.5)	37.4 (18.3-63.0)
Versican (% stained tissue)	43.9 (19.5-69.8)	35.4 (21.8-64.4)
Decorin (% stained tissue)	42.6 (20.5-85.3)	39.0 (14.7-55.4)

Data are presented as median (range). *: P<0.05

INFLAMMATORY CELLS IN INDUCED SPUTUM

Induced sputum samples were successfully obtained in 24 of our 37 asthmatic patients. Apart from eosinophils, total or differential cell counts were comparable (Table 4). Sputum eosinophil percentages were significantly higher in asthmatics with a positive PC₂₀AMP than with a negative PC₂₀AMP (Table 4, Figure 1G). Moreover, the percentage of sputum eosinophils correlated strongly with the AMP dose-response slope (Figure 1H).

TABLE 4: INFLAMMATORY CELLS IN INDUCED SPUTUM

	Negative PC ₂₀ AMP (n=17)	Positive PC ₂₀ AMP (n=20)
Sputum not evaluable (n (%))	5 (29)	8 (40)
No sputum (n (%))	3 (18)	3 (15)
>80% squamous (n (%))	2 (11)	3 (15)
<600 cells (n (%))	2 (11)	2 (10)
Sputum evaluable (n (%))	12 (71)	12 (60)
Total cells (x10 ⁶ /l)	0.3 (0.1-2.3)	0.3 (0.0-2.0)
Neutrophils (%)	56.8 (20.7-94.5)	56.5 (19.8-93.6)
Macrophages (%)	40.0 (5.3-76.4)	32.2 (5.6-76.2)
Eosinophils (%)	0.2 (0-1.8)	2.3 (0-25.7)*
Lymphocytes (%)	2.0 (0.2-6.9)	1.6 (0-5.9)

Data are presented as median (percentage or range). *: P<0.05

IMMUNOHISTOCHEMICAL LOCALIZATION OF A_{2A}, A_{2B} AND A₃ RECEPTORS IN BRONCHIAL BIOPSIES

To further unravel the mechanism of AMP responsiveness, we investigated the expression patterns of A_{2A}, A_{2B} and A₃ receptors. Of these receptors, the A_{2A} receptor showed the most abundant expression pattern, localized in the complete bronchial epithelial layer, the vascular endothelium, bronchial smooth muscle, and in infiltrating cells in the submucosal area (Figure 3A). The A_{2B} receptor was present mainly in basal cells of the bronchial epithelium, the bronchial smooth muscle, and in infiltrating cells in the submucosal area, many of which had an elongated shape suggestive of mast cells (Figure 3B). The A₃ receptor showed the least abundant expression pattern of the 3 receptors, with expression in the basal cells of the bronchial epithelium and bronchial smooth muscle (Figure 3C). The pattern of expression of these three adenosine receptors was not significantly different between the asthmatics with a positive and negative PC₂₀AMP (Table 5). We did not quantify the A₁ receptor since this receptor was nominal in bronchial biopsies.

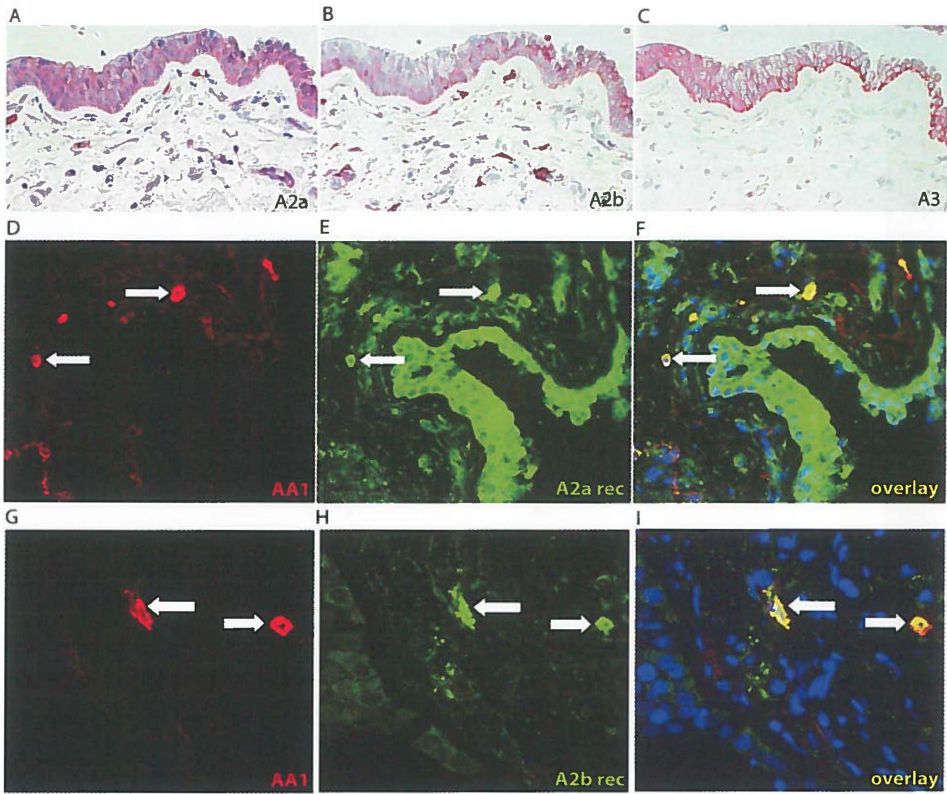


Figure 3

Adenosine receptor expression in bronchial biopsies. Representative pictures of A2a (A), A2b (B) and A3 receptor expression (C) in consecutive slides of the same biopsy sample. A2a receptor expression in mast cells was shown by fluorescent double-staining for AA1 (D) and A2a receptor (E). The overlap of signal is shown in the overlay (F). A2b receptor expression in mast cells was shown by fluorescent double-staining for AA1 (G) and A2b receptor (H). The overlap of signal is shown in the overlay (I). Arrows indicate examples of double-positive cells. Original magnification 400x.

EXPRESSION OF A2A AND A2B RECEPTORS ON MAST CELLS

Since A2a and A2b receptors on mast cells are thought to contribute to mast cell degranulation and AMP responsiveness, we studied mast cell expression of A2a and A2b receptors in a subset of patients by immunofluorescence and specific antibodies. Both A2a (Figure 3D-F) and A2b receptor (Figure 3G-I) expression was shown on a high percentage of AA1+ mast cells with medians ranging from 65 to 100% (Table 6). Double-staining for tryptase elucidated that the A2b receptor positive spindle-shaped cells were mast cells (Figure 3G-I). The percentages of A2a and A2b receptor positive mast cells in the submucosal area and smooth muscle were not significantly different between asthmatics with and without a positive PC20AMP (Table 6).

TABLE 5: ADENOSINE RECEPTOR EXPRESSION IN BRONCHIAL BIOPSIES

	Negative PC ₂₀ AMP N=16	Positive PC ₂₀ AMP N=13
<i>A2a receptor</i>		
Epithelium	1.25	1
Inflammatory cells	1	1.25
Smooth muscle	1	1
Vascular endothelium	1	1.5
<i>A2b receptor</i>		
Epithelium	1.5	1
Inflammatory cells	1.5	1.5
Smooth muscle	1	1
<i>A3 receptor</i>		
Epithelium	1.5	1.5
Smooth muscle	2	2

Expression was scored in a semi-quantitative way ranging from 0 to 2 (see methods section). Data are presented as median. No significant differences between the groups.

TABLE 6: ADENOSINE RECEPTOR EXPRESSION IN MAST CELLS

	Negative PC ₂₀ AMP	Positive PC ₂₀ AMP
<i>Submucosal</i>		
A2a ⁺ mast cells (%)	73 (56-84)	67 (58-90)
A2b ⁺ mast cells (%)	70 (42-87)	75 (70-78)
<i>Smooth Muscle</i>		
A2a ⁺ mast cells (%)	100 (0-100)	83 (19-100)
A2b ⁺ mast cells (%)	80 (67-100)	65 (50-74)

Data are presented as median (range). The cell numbers are expressed per 0.1 mm² of tissue. No significant differences between the groups.

DISCUSSION

This is the first study describing the pathological phenotype of AMP-responsive asthma patients, which appears to be characterized by airway wall eosinophilic inflammation and basement membrane thickening. Our data indicate that an AMP provocation test can be used to identify specific asthma phenotypes.

To our knowledge, we are the first to show that AMP responsiveness in asthma is associated with increased eosinophilic inflammation in the bronchial airway wall. Mechanistically, AMP is thought to exert its action via adenosine receptors. Since activation of the A₃ receptor on eosinophils has been described to inhibit their migration(17), it also would have been of interest to study the expression pattern of this receptor on eosinophils. The number of A₃-positive eosinophils in bronchial biopsy tissue were, however, too low for reliable quantification.

The increase in eosinophils in bronchial biopsies was paralleled by increased sputum eosinophil numbers in AMP-responsive asthmatics, compatible with results from previous studies(4;5;18;19) and indicating that sputum eosinophilia reflects airway wall inflammation in this group of patients. Migration of eosinophils to the sputum in asthma patients is a characteristic feature of AMP provocation(18;19), and does not occur after methacholine provocation. We have shown previously that an AMP-induced increase in sputum eosinophils is less pronounced in asthmatics using inhaled corticosteroids (ICS)(19), a drug specifically suppressing eosinophilic inflammation(20). Furthermore, we showed that both AMP responsiveness and sputum eosinophil numbers decrease after therapy with ICS(5;21). These findings support a causal relationship between AMP responsiveness and eosinophilic inflammation. Moreover, in a randomized, double-blind, crossover study on the changes in BHR with AMP and sputum cell counts in asthma patients after budesonide treatment, it was indeed shown that AMP-responsiveness reflects the anti-inflammatory effects of corticosteroids(22). A positive AMP provocation test may thus identify asthmatics that are responsive to ICS, thereby signifying the importance of identification of an AMP-responsive phenotype.

The second characteristic of the AMP-responsive phenotype in asthma was an increased BM thickness. Moreover, a thicker BM strongly correlated with more severe AMP responsiveness. This finding is in line with previous studies indicating not only a relationship between BM thickness and BHR assessed with AMP(23) provocation but also with methacholine(24-26) and histamine(27), suggesting an overall role for BM thickening in BHR. Although it is possible that airway structural changes, like BM thickening, contribute to airflow limitation or BHR, a direct causal relationship has not been shown so far.

BM thickness additionally strongly correlated with eosinophilic inflammation in bronchial biopsies and sputum, a finding consistent with the literature(24-26;28;29) and supporting a role for airway eosinophils in the process leading to BM thickening. Indeed, airway eosinophils have been shown to produce TGF- β (30), a well-established inducer of extracellular matrix (ECM) production by stimulating proliferation and differentiation of ECM producing cells, i.e. (myo)fibroblasts(31). This has been hypothesized to lead to excessive ECM deposition at the lamina reticularis, thereby thickening the airway subepithelial BM. Moreover, reduction of airway eosinophils after 12-months ICS treatment reverses BM thickening(25), also supporting the observed relation of airway eosinophils with BM thickening. A thickened BM is, however, not specific to asthma since this is also observed in other diseases with increased eosinophil numbers, like rhinitis and eosinophilic bronchitis without asthma symptoms(28). Therefore, we assume that BM thickening is an epiphenomenon of eosinophilic inflammation in asthmatics with AMP responsiveness rather than the underlying cause of AMP responsiveness itself.



Although several studies have shown that AMP-induced bronchoconstriction exerts its main action via the release of mast cell mediators, asthmatics with a positive PC20AMP in our study did not show higher submucosal mast cell numbers than those with a negative PC20AMP. It is conceivable that the smooth muscle rather than the bronchial submucosa is of relevance for AMP-induced bronchoconstriction, since mast cell degranulation products cause smooth muscle constriction. Nevertheless, we also did not find an association between AMP responsiveness and mast cell numbers in the smooth muscle. Our data are in agreement with two earlier studies in asthma that were not able to demonstrate an association between AMP responsiveness and mast cell numbers in both bronchial mucosa and smooth muscle (32;33). This lack of association may be due to the fact that the mast cells involved in bronchoconstriction reside in more peripheral parts of the lung rather than the central airways, which we studied. Indeed, the peripheral airways contain more mast cells in the airway wall and smooth muscle than central airways(14). Another option is that while there is not an increase in mast cell numbers between AMP-responsive and non-responsive asthmatics, the relevant issue is that induced changes in mast cell functionality are responsible for mast cell-mediated AMP responsiveness.

We investigated the functionality of mast cells by adenosine receptor expression, i.e. A2a and A2b receptors on mast cells, since A1 and A3 receptors are not expressed(2;34). Studies from human cultured mast cells showed that A2a receptor activation inhibits their histamine and tryptase release(35), while A2b receptor activation has an opposite effect, i.e. mast cell degranulation(36). Based on these findings we hypothesized that an unbalance in A2a/A2b receptor expression may accompany AMP responsiveness. Our data did, however, not confirm this hypothesis. We were unable to distinguish AMP-responsive and non-responsive asthmatics based on mast cell A2a and A2b receptor expression. We also did not find evidence for an association between AMP responsiveness and overall expression pattern of A2a, A2b and A3 receptors in asthmatic bronchial biopsies. We think this does not rule out a role for adenosine receptors in asthma, since differences are found in adenosine receptor expression on blood and sputum inflammatory cells between asthmatics and healthy controls and between samples before and after allergen challenge(37). Our data suggest that AMP responsiveness in asthma is not directly mediated by adenosine receptor expression. Clearly, functional studies using selective adenosine receptor agonists or antagonists are needed to prove or refute the contribution of adenosine receptors in AMP responsiveness in asthma.

In conclusion, we show that AMP responsiveness in asthma identifies asthmatics with a distinct pathological phenotype characterized by eosinophilic airway inflammation and BM thickening. As eosinophilic inflammation mostly responds well to corticosteroids, an AMP provocation test might be used to identify corticosteroid responsive asthmatics. This is an interesting option that has to be proven in future studies.

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AMP responsive asthma is characterized by eosinophils and basement membrane thickening

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Online data supplement

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IMMUNOHISTOCHEMICAL STAINING PROCEDURES

In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. CD3, CD4, CD20, NP57, CD68, AA1, decorin and versican were detected with Envision™ Detection Kit (DAKO). Ki67, E-cadherin and collagen 3 were detected with two peroxidase-labelled conjugates (both DAKO). CD8 and EPX were detected with biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham AL, USA) and streptavidine-peroxidase conjugate (CD8) or streptavidine-alkaline phosphatase conjugate (EPX, both DAKO). Adenosine receptors were detected with biotinylated anti-rabbit conjugate (Southern Biotechnology) and strepABComplex/PO (DAKO). We chose the chromogen for each antigen based on its suitability for quantification, i.e. NovaRED for CD3, CD4, CD8, CD20, NP57, CD68 and AA1 (Vector Labs, Burlingame, USA), 3,3'-Diaminobenzidine tetrahydrochloride for decorin, versican, collagen 3, Ki67 and E-cadherin (DAB, Sigma-Aldrich, Zwijndrecht, the Netherlands), Permanent Red for EPX (DAKO) or 3-amino-9-ethylcarbazole for Adenosine Receptors (AEC, Sigma-Aldrich).

Immunofluorescence double staining was performed to show adenosine A2a and A2b receptor expression on mast cells, using donkey anti-rabbit ALEXA 488 (Invitrogen, Breda, the Netherlands) for the receptors and donkey anti-mouse ALEXA 555 (Invitrogen) for mast cells. Counterstaining was performed with 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Fluorescence microscopy was performed using a Leica DMLB microscope (Leica Microsystems, Rijswijk, the Netherlands), Leica DC300F camera and Leica QWin 2.8 software.

Airway Eosinophilia in Remission and Progression of Asthma: Accumulation with a Fast Decline of FEV₁

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ABSTRACT

Background: As it is unknown whether complete asthma remission or progression of asthma is associated with airway inflammation and remodeling, we assessed these characteristics in bronchial biopsies of relevant subsets of asthma patients.

Methods: Sputum and bronchial biopsies were obtained from asthma patients in remission (PC₂₀ histamine >32 mg/ml, PC₂₀ AMP >320 mg/ml) and from those with either a slow FEV₁ decline (<30 ml/year) or fast decline (>30 ml/year). Inflammatory cells and mediators were determined in sputum, inflammatory cells and aspects of airway remodeling in bronchial biopsies.

Results: Asthmatics in remission and asthma patients with a slow FEV₁ decline had a similar extent of airway inflammation and remodeling in sputum and bronchial biopsies. Asthma patients with a fast FEV₁ decline had high sputum eosinophil numbers. Moreover, FEV₁ decline (ml/year) correlated with sputum eosinophil numbers ($R_s=0.51$, $P=0.003$) and ECP levels ($R_s=0.57$, $P=0.001$). Airway remodeling, i.e. basement membrane thickness, correlated with sputum eosinophils ($R_s=0.69$, $p<0.001$), sputum ECP ($R_s=0.46$, $p=0.018$) and airway wall eosinophil numbers ($R_s=0.49$, $p=0.002$).

Conclusions: Asthma, even when in remission, is accompanied by airway inflammation and remodeling. Data suggest that eosinophils are important in a subset of asthma patients by association to accelerated FEV₁ decline and change of basement membrane thickness.

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways associated with airway obstruction that is often reversible, either spontaneously or with treatment. Nevertheless, asthma patients have on average a lower lung function than healthy controls (1). Furthermore, some patients have an even accelerated decline in forced expiratory volume in 1 second (FEV₁) over time (2), probably caused by ongoing airway inflammation and the resulting airway remodeling (3). Not all asthmatics are predestined for this scenario since some subjects show stable lung function and others even outgrow their disease. As yet, it is unknown whether these differences in the natural course of clinical asthma reflect differences in the underlying inflammatory processes and structural changes in the airways.

Some patients fully outgrow their asthma, so-called asthma remission, which is characterized by normal lung function, absence of bronchial hyperresponsiveness (BHR) and respiratory symptoms while not using asthma medication (4). Others are in "clinical" asthma remission, i.e. they are asymptomatic but still show BHR. The latter type of remission is associated with ongoing airway inflammation and remodeling as reflected by increased numbers of eosinophils, T cells and mast cells in the airway mucosa and thickening of the reticular basement membrane (BM) when compared to control subjects without asthma (5). However, it is unknown whether complete remission with the absence of BHR is associated with less airway inflammation and remodeling when compared to persistent asthma. Therefore, we investigated airway inflammation and remodeling in patients with complete asthma remission, defined by the absence of BHR, asthma symptoms and treatment.

Previous studies have revealed multiple risk factors for an accelerated FEV₁ decline (15–50 ml/year) in asthma: low baseline lung function (FEV₁ % predicted) (6), less reversibility to β_2 -agonists (6), more severe BHR (7), smoking (8), increased mucus production (2), male gender (9) and frequent exacerbations (10). The pathological processes underlying the accelerated FEV₁ decline in asthma are unknown. Several suggestions for the underlying pathological process come from cross-sectional studies demonstrating an association between the above described risk factors and markers of airway inflammation and remodeling. For example, low baseline lung function has been associated with increased airway wall thickness (11), more severe BHR with thickening of the subepithelial BM (12), smoking with epithelial metaplasia (13), goblet cell hyperplasia (14) and increased subepithelial collagen deposition (15), and increased mucus production has been associated with hyperplasia and hypertrophy of epithelial goblet cells (16) and increased presence of submucosal mucous glands (16). Moreover, Ulrik et al. (17) showed an association between higher blood eosinophil numbers and accelerated FEV₁ decline in adult asthma patients, thereby postulating a role of eosinophils. Based on these findings it is tempting to speculate that an accelerated FEV₁ decline in asthma indeed results from progressive airway inflammation and remodeling.

Aim of this study was to characterize the pathological background of asthma remission and progression. Therefore, we recruited 47 steroid-naïve, non-smoking subjects with a documented asthma diagnosis in the past, we assessed their annual FEV₁ decline over a minimum period of at least 5 years and categorized them into 3 groups: subjects with asthma remission, current asthma patients with a slow FEV₁ decline (<30 ml/year) and a fast FEV₁ decline (>30 ml/year). Airway inflammation and remodeling were investigated in sputum samples and bronchial biopsies.

METHODS

SUBJECTS

Non-smoking subjects (n=47) aged between 32 and 70 years, without oral or inhaled corticosteroids were recruited. All subjects originated from research cohorts investigated earlier by our research group and all had a doctor's diagnosis of asthma and a documented PC20 histamine (using 30-seconds tidal breathing) \leq 32 mg/ml in the past (4). These

subjects were extensively characterized according to a standard protocol (including FEV₁) at least five years prior to our study. Subjects were re-assessed in the present study with lung function, hyperresponsiveness to adenosine 5'-monophosphate (AMP), sputum induction and bronchial biopsies. Three groups were identified: 1) asthma remission (FEV₁ >90% predicted, PC20 AMP >320 mg/ml, PC20 histamine >32 mg/ml), 2) slow FEV₁ decline (<30 ml/year over at least 5 years and PC20 AMP ≤320 mg/ml or PC20 histamine ≤32 mg/ml), 3) fast FEV₁ decline (>30 ml/year over at least 5 years and PC20 AMP ≤320 mg/ml or PC20 histamine ≤32 mg/ml). Main exclusion criteria were: the use of corticosteroids during the period over which the FEV₁ decline was being calculated or more than 5 years of cumulative corticosteroid use in the distant past. Furthermore, FEV₁ <1.2 L, bronchiectases, upper respiratory tract infection (e.g. colds) and/or use of antibiotics the last 2 months before inclusion were exclusion criteria. The study protocol was approved by the local medical ethics committee. All subjects gave their written informed consent.

STUDY DESIGN

Peripheral blood eosinophil counts and IgE, induced sputum samples and lung function (flow-volume, CO diffusion) were obtained. An AMP provocation test was performed followed 1-2 weeks later by a bronchoscopy. Subjects with a negative AMP provocation test (PC20 AMP >320 mg/ml) were subjected to a histamine provocation test >3 weeks later to determine BHR.

LUNG FUNCTION

FEV₁ was measured with a calibrated water-sealed spirometer at the current and past visit (Lode Spirograph D53, Lode Instruments, Groningen, the Netherlands). Reversibility of FEV₁ (% predicted) was measured after administration of 800 µg of Albuterol. Annual FEV₁ decline (mL/year) was calculated by subtracting FEV₁ (post-bronchodilator) at the first and second measurement, divided by the number of years between the two measurements (minimum of 5 years). Provocation tests were performed with a method adapted from Cockcroft and coworkers (18). After an initial nebulized 0.9% saline challenge, subjects inhaled doubling concentrations AMP (0.04 to 320 mg/ml) by 2-min tidal breathing, at 5-min intervals. BHR to histamine was tested as reported previously (19), using 30-seconds tidal breathing and doubling concentrations ranging from 0.13 to 32 mg/ml.

ATOPY MEASUREMENTS

Atopy was determined by the Phadiatop screening test with the ImmunoCap system (Phadia AB, Sweden) and results are presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Positive Phadiatop was defined as patient serum/control serum >1.

SPUTUM INDUCTION AND PROCESSING

Sputum was induced by inhalation of 5% hypertonic saline aerosols as over 3 consecutive periods of 5 min. Processed whole sputum samples were stained with May Grünwald Giemsa (MGG) to obtain cell differentials by counting total 600 viable, non-squamous cells. Sputum was not scored if the percentage squamous cells was >80% or the total number of non-squamous cells was <600.

SPUTUM SUPERNATANT MEASUREMENTS

Histamine was measured by ELISA (IBL, Hamburg, Germany). Adenosine was measured by HPLC as described previously (20). The concentration of eosinophilic cationic protein (ECP) was measured using a fluoroenzyme immunoassay (ImmunoCAP ECP, Pharmacia, Uppsala, Sweden).

Secreted mucin was measured by double-sandwich ELISA (Covance, Emeryville, USA), with the 17B1 protein-G purified antibody and 17Q2 alkaline phosphatase-conjugated antibody specifically binding to the granules of the surface goblet cells and mucous

gland cells, as previously described (21).

Collection, processing and immunohistochemical staining of bronchial biopsies
After local anaesthesia, bronchial biopsies were obtained using a flexible bronchoscope (type Olympus BF P20 or BF XT20) from segmental divisions of the main bronchi. The biopsies were fixed in 4% formalin, processed and embedded in paraffin. Bronchial biopsies were cut in 3 µm thick sections. All stainings were performed in an automated system using the DAKO autostainer and were manually counterstained with methylgreen.

The inflammatory profile was assessed with specific antibodies against eosinophilic peroxidase (EPX, the laboratories of Drs. NA Lee and JJ Lee), mast cell tryptase (AA1, DAKO, Glostrup, Denmark), macrophages (CD68, DAKO), neutrophil elastase (NP57, DAKO) and T-lymphocytes (CD3 (DAKO), CD4 (Novocastra, Newcastle upon Tyne, UK) and CD8 (DAKO)). In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) except for EPX and CD8, which were detected using biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham AL, USA) and alkaline phosphatase- (DAKO) or peroxidase-labelled streptavidine conjugates (DAKO). Permanent Red (DAKO) or NovaRED (Vector Labs, Burlingame, USA) was used as chromogen.

Periodic acid-Schiff (PAS) staining was used to determine the presence of goblet cells in the bronchial epithelium. Haematoxylin eosin (HE) staining was used for evaluation of epithelial integrity and basement membrane (BM) thickness.

Deposition of extracellular matrix (ECM) components in the sub-epithelial compartment was assessed by specific antibodies against collagen 3 (Southern Biotechnology), decorin (dermatan sulfate proteoglycan, Seikagaku, Tokyo, Japan) and versican (large proteoglycan (versican), Seikagaku). In short, sections were deparaffinized and after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) except for collagen 3, which was detected using peroxidase labelled rabbit anti-goat and goat anti-rabbit conjugates (both DAKO). 3,3'-Diaminobenzidine tetrachloride (DAKO) was used as chromogen.

QUANTIFICATION OF THE HISTOLOGICAL STAININGS

Quantification of inflammatory cells was performed using computerized-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). A blinded investigator counted the number of positively stained inflammatory cells in the submucosal area. This submucosal area was restricted to 100µm under the basement membrane (BM) and a total area of 0.1mm². For each subject three sections of one biopsy sample were counted and average numbers were used for further analysis.

The number of goblet cells was counted on PAS-stained biopsy sections and expressed per mm of BM. Epithelial integrity was determined using HE-stained biopsy sections and was expressed as the percentage of BM that was covered with either intact epithelium (a layer of basal and ciliated columnar epithelial cells without detachment from the BM), only basal epithelial cells, metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells) or the percentage of BM that was denuded from epithelial cells. BM thickness was determined by the measurement of the total BM length and surface on HE-stained biopsy sections. Subsequently, the mean thickness could be calculated by dividing the BM surface area by its length.

Expression of ECM components was measured by computerized-assisted image analysis in the submucosal area 200 µm under the BM, and was expressed as the percentage of positive tissue per biopsy section. For each subject two different biopsy sections were quantified and average percentages were used for further analysis.

STATISTICS

All analyses were performed using SPSS (version 14.0; SPSS Inc, Chicago, IL, USA). Normality of distributions was assessed using the Kolmogorov-Smirnov test. When

necessary, normalization by transformation was attempted. Normally distributed data was analysed with one-way ANOVA and independent samples t-tests. Non-normally distributed data was analysed with Kruskal-Wallis and Mann-Whitney U tests. Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson (for normally distributed data) or Spearman (for non-normally distributed data) tests. Two tailed p-values of <0.05 were considered statistically significant.

RESULTS

SUBJECT CHARACTERISTICS

Forty-seven subjects were included: 10 subjects with complete asthma remission, 20 asthma patients with a slow FEV₁ decline over at least 5 years (<30 mL/year) and 17 with a fast FEV₁ decline over at least 5 years (>30 mL/year). As expected, FEV₁ was lowest in asthmatics with a fast FEV₁ decline, intermediate with a slow decline and highest in asthma remission; reversibility was not significantly different between the three groups. Asthma patients with a fast FEV₁ decline had significantly higher blood eosinophil counts than those with a slow FEV₁ decline (Table 1).

TABLE 1: PATIENT CHARACTERISTICS

	Remission (n=10)	Slow FEV ₁ Decline (n=20)	Fast FEV ₁ Decline (n=17)
Sex (M/F)	3/7	11/9	12/5
Age (yr)	53 (32-67)	48 (35-70)	53 (38-64)
FEV ₁ decline (ml/year)	8.0 (-35-35)	18.6 (-27-29)	50.3 (34-105) ^{#,§}
Interval between FEV ₁ measurements (years)	8.7 (6-14)	8.9 (5-13)	9.3 (5-13)
FEV ₁ (% predicted)	111 (102-146)	100 (78-131)*	98 (77-127) [#]
FEV ₁ /VC (%)	79 (67-93)	75 (65-91)	73 (56-84) [#]
MEF ₅₀ (% predicted)	105 (60-147)	69 (46-147)*	68 (36-98) [#]
Reversibility FEV ₁ (%)	3.9 (0.4-11.5)	8 (-0.4-28.7)	8.3 (-8.4-17.2)
PC ₂₀ AMP (mg/ml)	>320	>320 (0.02->320)*	46.2 (0.02->320) [#]
KCO (% predicted)	95 (84-131)	99 (78-127)	110 (82-141)
IgE (IU/L)	60 (7-231)	66 (14-552)	70 (11-558)
Positive Phadiatop (n (%))	5 (50%)	13 (65%)	13 (76%)
Phadiatop score (ratio)	0.96 (0.33-54)	9.1 (0.22-92)	15 (0.06-92)
Blood eosinophils (x10 ⁹ /L)	0.12 (0.06-0.24)	0.12 (0.02-0.35)	0.19 (0.07-0.51) [§]

Values are presented as median values (ranges), unless stated otherwise. All spirometric data were determined after inhalation of 800 µg of Albuterol; reversibility FEV₁ = change in FEV₁, expressed as increase in percentage predicted value after 800 µg of Albuterol; PC₂₀ AMP = provocative concentration of adenosine 5' monophosphate causing a 20% fall in FEV₁; Positive Phadiatop = specific IgE's in patient serum/control serum >1; n = number.

* p<0.05 remission vs. slow FEV₁ decline

[#] p<0.05 remission vs. fast FEV₁ decline

[§] p<0.05 slow vs. fast FEV₁ decline.

INFLAMMATORY CELLS AND INFLAMMATORY MEDIATORS IN SPUTUM

The absolute number of sputum eosinophils was higher in patients with a fast FEV₁ decline than with a slow FEV₁ decline (Table 2, Figure 1A). Annual FEV₁ decline correlated significantly with the absolute number of sputum eosinophils (Figure 1B). Sputum neutrophils, macrophages and lymphocytes did not significantly differ between the three study groups (Table 2).

The levels of inflammatory mediators in the supernatant of induced sputum, i.e. histamine, adenosine and mucin were similar for subjects with asthma remission and asthma patients with a slow or a fast FEV₁ decline (Table 2). Moreover, the levels of inflammatory cytokines and growth factors (IFN- γ , IL-4, 5, 8, 13, 17, EGF, Eotaxin, FGFb, GM-CSF, MCP1, RANTES, VEGF) were similar for all groups (See additional file 1). Sputum ECP values were highest in asthma patients with a fast FEV₁ decline (59.8 (12-2467) μ g/l), values being significantly higher than in subjects with asthma remission (16.0 (3-172) μ g/l) and slightly, but not significantly, higher than in asthma patients with a slow FEV₁ decline (27.0 (6-447) μ g/l, $p=0.056$) (Table 2, Figure 1C). Moreover, a higher annual FEV₁ decline correlated significantly with higher sputum ECP levels (Figure 1D).

TABLE 2: INFLAMMATORY CELLS AND MEDIATORS IN SPUTUM

	Remission (n=10)	Slow FEV ₁ Decline (n=20)	Fast FEV ₁ Decline (n=17)
No sputum	2 (20%)	3 (15%)	3 (18%)
Sputum not evaluable			
>80% squamous n (%)	1 (10%)	2 (10%)	3 (18%)
<600 cells n (%)	-	-	2 (11%)
Total cells (x10 ⁶ /ml)	0.46 (0.06-2.90)	0.31 (0.12-2.27)	0.41 (0.03-2.05)
Neutrophils (%)	62 (19-78)	60 (20-94)	55 (35-85)
(x10 ⁶ /ml)	0.40 (0.03-1.19)	0.21 (0.03-1.58)	0.28 (0.11-1.44)
Macrophages (%)	36 (20-78)	32 (5-76)	33 (13-62)
(x10 ⁶ /ml)	0.16 (0.02-2.14)	0.10 (0.01-1.07)	0.18 (0.08-0.61)
Eosinophils (%)	0.2 (0-4.6)	0.2 (0-4.8)	1.8 (0-26.0)
(x10 ⁶ /ml)	0.002 (0-0.03)	0.001 (0-0.02)	0.01 (0-0.34)*
Lymphocytes (%)	2.2 (1.4-7.3)	2.6 (0.2-6.9)	1.3 (0-5.6)
(x10 ⁶ /ml)	0.02 (0.001-0.04)	0.01 (0.001-0.07)	0.004 (0-0.11)
Histamine (ng/ml)	13.4 (2.8-29.8)	9.1 (0.2-28.8)	13.8 (4.8-38.0)
Adenosine (ng/ml)	56.5 (8.0-110)	34.6 (6.2-178)	23.0 (0.9-201)
ECP (μ g/l)	16.0 (2.5-172)	27.0 (6.0-447)	59.8 (11.9-2467)* [#]
Mucin (units)	0.25 (0.04-3.9)	0.78 (0.08-10.3)	1.0 (0.18-2.7)

Values are presented as median values (percentages or ranges). Eosinophils: * $p<0.05$ vs. slow FEV₁ decline. ECP: * $p=0.056$ vs. slow FEV₁ decline, [#] $p<0.05$ vs. remission. For luminex data see Table S1.

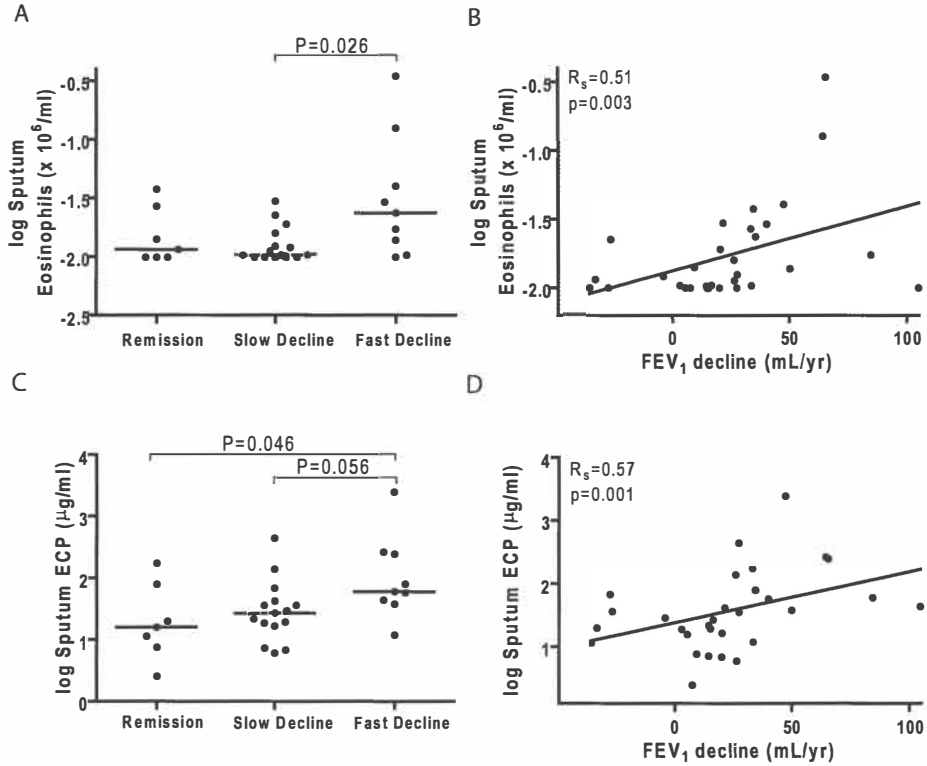


Figure 1

Eosinophilic inflammation in sputum. (A) Absolute number of sputum eosinophils (log transformed) in subjects with asthma remission and asthmatic subjects with a slow or a fast FEV₁ decline. (B) Correlation between the annual FEV₁ decline and the absolute number of sputum eosinophils (log transformed). (C) Sputum ECP (log transformed) in subjects with asthma remission and asthmatic subjects with a slow or a fast FEV₁ decline. (D) Correlation between the annual FEV₁ decline and sputum ECP (log transformed). Each closed circle represents one subject. Horizontal bars represent median values.

INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

No significant differences were observed in numbers of eosinophils, mast cells, macrophages, neutrophils and T lymphocytes in bronchial biopsies of subjects with asthma remission, asthma patients with a slow or fast FEV₁ decline (Table 3, Figure 2A-C).

TABLE 3: INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

	Remission (n=9)	Slow FEV ₁ Decline (n=18)	Fast FEV ₁ Decline (n=11)
EPX ⁺ eosinophils	4 (0-10)	3 (0-18)	3 (0-40)
AA1 ⁺ mast cells	5 (1-20)	3 (0-13)	7 (1-19)
CD68 ⁺ macrophages	17 (12-86)	16 (4-37)	17 (3-30)
NP57 ⁺ neutrophils	9 (2-30)	9 (1-46)	9 (2-38)
CD3 ⁺ lymphocytes	75 (35-291)	77 (21-177)	62 (33-246)
CD4 ⁺ lymphocytes	19 (9-168)	21 (2-67)	24 (9-65)
CD8 ⁺ lymphocytes	28 (7-112)	21 (1-112)	17 (3-103)

Number of cells expressed per 0.1 mm². Values are presented as median values (ranges). No significant differences between the 3 groups.

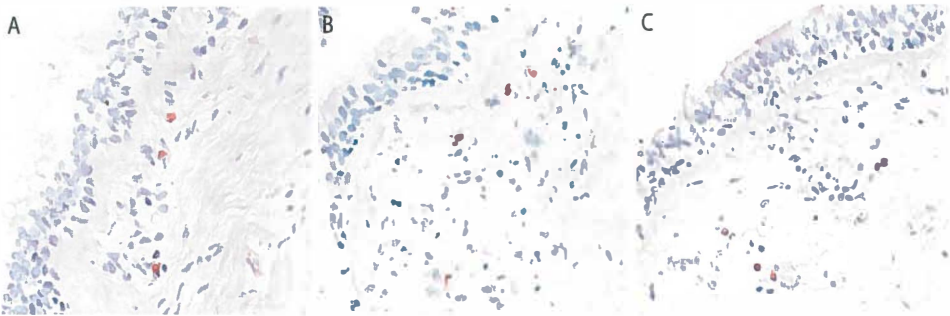


Figure 2
EPX⁺ eosinophils in bronchial biopsies. Representative pictures of EPX⁺ eosinophils in biopsies of subjects in (A) asthma remission and asthmatic patients with a (B) slow FEV₁ decline or a (C) fast FEV₁ decline. Lens magnification 400x.

AIRWAY REMODELING DETERMINED IN BRONCHIAL BIOPSIES

No differences were observed in remodeling parameters in bronchial epithelium (goblet cells and epithelial integrity), BM (thickness) and submucosal tissue (presence of ECM components) between subjects with asthma remission and asthma patients with a slow or fast FEV₁ decline (Table 4).

BM thickness correlated significantly with all investigated parameters in sputum and bronchial biopsies that are associated with eosinophilic inflammation, i.e. sputum eosinophils (Figure 3A), sputum ECP (Figure 3B) and EPX+ cells in biopsies (Figure 3C). The correlation scatters showed two outliers for BM thickness (Figure 3). However, without these two subjects, significant correlations were still present between BM thickness and all investigated eosinophil markers (not shown).

TABLE 4: REMODELING IN BRONCHIAL BIOPSIES

		Remission (n=9)	Slow FEV₁ Decline (n=18)	Fast FEV₁ Decline (n=11)
Epithelium	Goblet cells (N)	28 (0-42)	36 (7-78)	22 (4-220)
	Intact epithelium (%)	6.8 (0-32)	6.8 (0-30)	6.7 (0-34)
	Basal epithelium (%)	63 (30-82)	59 (21-100)	50 (13-86)
	Denuded BM (%)	22 (0-48)	8 (0-52)	13 (0-59)
	Metaplasia (%)	0 (0-21)	0 (0-26)	0 (0-19)
Basement Membrane	BM thickness (μm)	6.3 (4.9-8.4)	6.6 (3.8-8.6)	7 (4.4-12.6)
Submucosa	Versican (%)	45 (20-67)	38 (19-70)	44 (29-69)
	Collagen 3 (%)	39 (15-48)	36 (17-63)	40 (9-65)
	Decorin (%)	43 (16-76)	36 (15-85)	44 (27-55)

Values are presented as median values (ranges). Intact and basal epithelium, denuded basement membrane (BM) and metaplasia are presented as the percentage of BM covered with this type of epithelium. Versican, collagen 3 and decorin are presented as the percentage of positive tissue. No significant differences between the 3 groups.

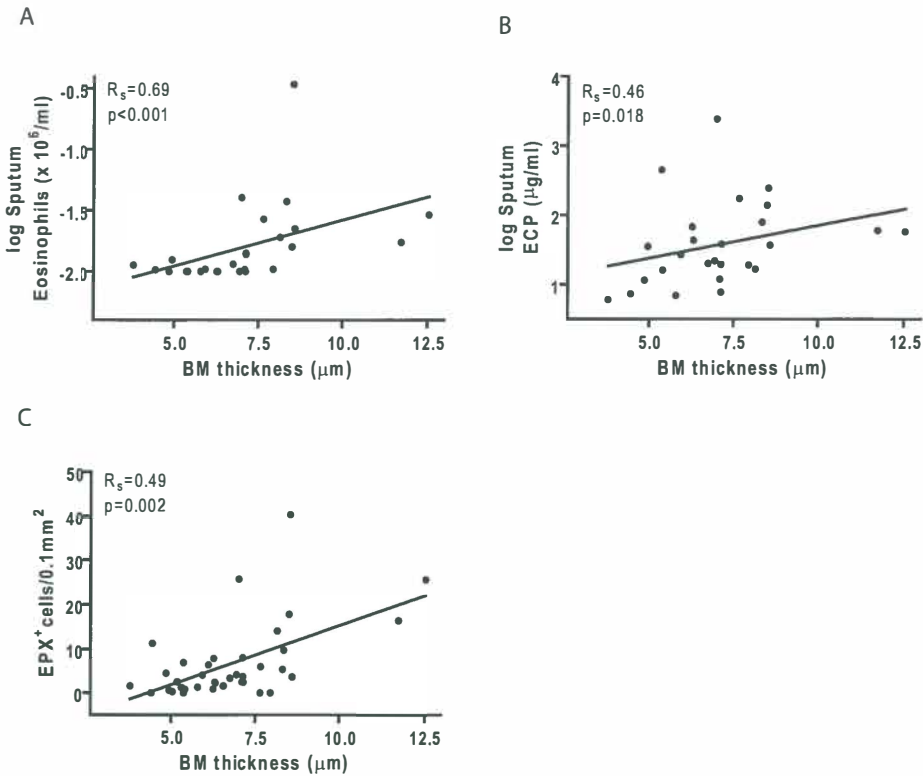


Figure 3

Eosinophilic inflammation and basement membrane thickness. (A) Correlation between basement membrane (BM) thickness and the absolute number of sputum eosinophils (log transformed). (B) Correlation between BM thickness and sputum ECP (log transformed). (C) Correlation between BM thickness and the number of EPX⁺ cells in 0.1mm^2 bronchial biopsy tissue. Each closed circle represents one subject.

DISCUSSION

The present study describes the pathological background of asthma remission and progression in a well-characterized group of asthma patients. We investigated patients with complete asthma remission, i.e. individuals with established asthma in the past who at the present time of investigation lacked asthma symptoms, airway obstruction and bronchial hyperresponsiveness (BHR) while not using any asthma treatment. This remission was associated with a similar extent of inflammation and remodeling as present in individuals with current asthma and an FEV₁ decline in the range of the normal population. In contrast, patients with current asthma and a fast FEV₁ decline had more pronounced eosinophilic inflammation in blood and sputum. In line with this observation, the annual FEV₁ decline correlated significantly with eosinophilia in sputum. Basement membrane (BM) thickness, which is considered as an important hallmark of airway remodeling, was positively correlated with eosinophilic inflammation in both sputum and the airway wall.

An important finding of our study is that these patients with complete asthma remission have signs of airway wall inflammation and remodeling. To our surprise, the extent of airway inflammation and remodeling is compatible with that of asthma patients with a slow FEV₁ decline. The question remains how to explain the similar findings in these two different groups of asthma patients. One possible explanation is that the clinical disease activity is not determined by the number but rather the activation state of inflammatory cells, and that complete asthma remission does not exclude the presence of quiescent inflammatory cells. However, our data shows that sputum levels of inflammatory cytokines and growth factors were similar in subjects with remission and current asthma, suggesting that the activation status and functionality of the inflammatory cells is not different. Another explanation may be that clinical disease activity is determined by inflammation and/or remodeling in other regions of the lung not accessible by induced sputum or bronchial biopsies. The type of clinical expression may for example be caused by the volume of smooth muscle in more distal parts of the central or peripheral airways, or the presence or absence of activated mast cells between smooth muscle bundles.

Our findings extend observations in other studies since ongoing airway inflammation and remodeling were also reported in subjects with less strict criteria for asthma remission (5;22), i.e. with BHR still present. It is still possible that these types of asthma patients need anti-inflammatory treatment. This is an intriguing yet unresolved question since no study has prospectively followed these patients to older age.

Our second interesting observation is that an accelerated FEV₁ decline is associated with increased sputum eosinophil numbers and ECP concentrations. This finding extends results of several cross-sectional studies demonstrating the relationship between eosinophilic inflammation and airway obstruction in asthma (23;24) and a longitudinal study showing a positive association between blood eosinophils and FEV₁ decline in asthma (17). However, FEV₁ decline in our asthma patients was not associated with the number of airway wall eosinophils, just as van Rensen et al. (25) found. There are two possible explanations for this discrepancy. First, bronchial biopsies give only a snap-shot of the cellular composition of the airway wall which is not identical to the traffic of inflammatory cells through the airway wall. Second, an accelerated FEV₁ decline may be associated with increased recruitment of eosinophils from the bone marrow to the blood. Possibly, these eosinophils move straight from the blood to the luminal space of the lung and are not retained in the airway wall e.g. due to epithelial damage.

Although not demonstrated by our study, it can be anticipated that additional aspects of inflammation, besides eosinophils, contribute to the FEV₁ decline in asthma as well. Previous studies already have shown an association between lower FEV₁ levels and neutrophilic inflammation in sputum (26) and between accelerated FEV₁ decline and CD8-positive T-cells in bronchial biopsies (25). However, these studies did, in contrast

to the "clean population" in our study, include asthma patients who smoked and/or used ICS, and it is known that smoking and ICS use change the inflammatory profile (19;27).

Our study did not find significant associations between FEV₁ decline and different remodeling parameters like mucus secretion in sputum and goblet cells in bronchial epithelium. Lange et al. reported an association between self-reported mucus production and FEV₁ decline (2). The fact that the latter study was a large epidemiological study over many years including a heterogeneous group of smoking and non-smoking asthmatics may explain the difference with our study.

Thickening of the subepithelial BM as a result of ECM deposition is one of the hallmarks of airway remodeling (28) and was previously shown to be negatively correlated with the level of FEV₁ in asthma (29). We did not observe an association between BM thickness and the annual FEV₁ decline, which is in line with observations by van Rensen et al (25). It has to be realized that BM thickening in asthma is considered to constitute an early airway wall change, already observed in childhood asthma (30). This may possibly explain why we did not observe an association with FEV₁ decline over the last years.

Importantly, BM thickness correlated significantly with sputum eosinophils, ECP concentrations in sputum and eosinophils in bronchial biopsies, but not with FEV₁. Indeed airway eosinophils have been shown to produce TGF- β (31) whereas ECP stimulates secretion of TGF- β by human lung fibroblasts (32). TGF- β is a well-established inducer of ECM production by stimulating the proliferation and differentiation of ECM producing cells, i.e. (myo)fibroblasts (33). This can lead to excessive ECM deposition at the lamina reticularis and thus thickening of the airway subepithelial BM. So far it is unclear whether thickening of the BM is an epiphenomenon or really contributes to the accelerated FEV₁ decline.

We realize that our study has both strengths and weaknesses. A strength is that we defined asthma remission by the additional criterion of the absence of BHR for both AMP and histamine. Thus our definition of asthma remission is more strict than the classical description of asthma remission (5;22), and it is better distinguishable from current asthma. Another strength is that we carefully excluded subjects with a history of smoking and use of (oral or) inhaled corticosteroids since ICS are known to reduce (27) and smoking to accelerate FEV₁ decline in asthma (19). The consequence of this exclusion may be that we included less severe asthma patients, thereby underestimating the differences in inflammation and remodeling. However, the obvious advantage of this exclusion is that our population enabled us to study the natural course of clinical asthma without these important confounding factors. A relative weakness of our study is that, although we studied FEV₁ longitudinally in our patients, we only sampled sputum and bronchial biopsies at the end of follow-up. Therefore, we can not exclude the possibility that eosinophilic inflammation in sputum is not the cause but rather the consequence of accelerated FEV₁ decline. Second, it can be argued that for an accurate estimation of longitudinal decline in lung function, two measurements, with an average interval of 8.9 years, are not sufficient (34). However, we would like to emphasize that the two spirometric measurements in our study were performed according to a standardized protocol using the same equipment.

The present study is of clinical relevance since it suggests the need for specific adaptations in therapy in subsets of asthma patients. First, we showed that subjects with complete asthma remission have airway inflammation. This observation does not support but also not refutes the recommendation to stop the use of ICS in these subjects. Long-term studies may give a definite answer on the question whether airway inflammation ultimately disappears completely. Second, we showed that asthma patients with a fast FEV₁ decline are characterized by increased eosinophilic

inflammation in blood and sputum. Therefore, intensive ICS treatment, known to reduce the accelerated FEV₁ decline (27), might be especially beneficial for asthmatics with increased eosinophilic inflammation.

In conclusion, complete clinical and functional asthma remission is accompanied by airway inflammation and remodeling. This indicates that there is no strong relationship between airway inflammation and BHR and, more importantly, that asthma remission never is really "complete". Furthermore, eosinophilic inflammation may be important in a subset of asthma patients as eosinophils are associated with accelerated FEV₁ decline and increased BM thickness.

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Airway eosinophilia in remission and progression of asthma: accumulation with a fast decline of FEV₁

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Online data supplement

5

LEVELS OF INFLAMMATORY CYTOKINES AND GROWTH FACTORS IN SPUTUM SUPERNATANT

	Remission (n=7)	Slow FEV ₁ Decline (n=15)	Fast FEV ₁ Decline (n=9)
IFN- γ (pg/ml)	11.4 (0-13.2)	11.4 (0-15.9)	13.2 (0-34.1)
IL-4 (pg/ml)	25.9 (0-41.4)	25.9 (0-72.3)	23.0 (0-40.4)
IL-5 (pg/ml)	4.0 (0-6.3)	4.0 (0-20.9)	5.0 (0-7.7)
IL-8 (pg/ml)	868.3 (78.0-3608)	486.4 (115-55167)	2384.5 (187-23666)
IL-13 (pg/ml)	0 (0-122)	0 (0-206)	0 (0-65.1)
IL-17 (pg/ml)	0 (0-65.9)	0 (0-106)	0 (0-94.0)
EGF (pg/ml)	46.3 (12.2-130)	64.0 (15.2-132)	92.6 (21.9-149)
Eotaxin (pg/ml)	0.3 (0-3.3)	2.1 (0-15.0)	0 (0-1.9)
FGFb (pg/ml)	0 (0-13.3)	0 (0-7.1)	0 (0-4.9)
GM-CSF (pg/ml)	94.3 (0-104)	94.3 (0-152)	94.3 (0-98.5)
MCP-1 (pg/ml)	319 (0-375)	347 (50.9-8620)	391 (195-922)
RANTES (pg/ml)	7.9 (0-13.1)	9.5 (0-21.0)	4.2 (0-16.9)
VEGF (pg/ml)	101 (46.0-156)	104 (53.0-252)	131 (51.8-249)

Values are presented as median values (ranges). No significant differences between the groups.

A human custom multiplex antibody bead kit (Biosource, Invitrogen Ltd, Paisley, UK) was used to measure IFN- γ , IL-4, 5, 8, 13, 17, EGF, Eotaxin, FGFb, GM-CSF, MCP1, RANTES, VEGF by Luminex, according to the manufacturers' instructions. Data was analysed using Starstation software. For each cytokine or growth factor undetectable values, those below the detection limit, were set at 0 (detection limits: IFN- γ : 5, IL-4: 5, IL-5: 3, IL-8: 3, IL-13: 10, IL-17: 10, EGF: 25, Eotaxin: 5, FGFb: 20, GM-CSF: 15, MCP1: 10, RANTES: 15, VEGF: 15 pg/ml).

We detected no significant differences in the level of the inflammatory cytokines and growth factors IFN- γ , IL-4, 5, 8, 13, 17, EGF, Eotaxin, FGFb, GM-CSF, MCP1, RANTES and VEGF between subjects with asthma remission and asthma patients with a slow or a fast FEV₁ decline.

Airway Epithelial Changes in Smokers but Not in Ex-Smokers with Asthma

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ABSTRACT

Rationale: Smoking has detrimental effects on asthma outcome, such as increased cough, wheezing, sputum production, and frequency of asthma attacks. This results in accelerated lung function decline. The underlying pathological process of smoke-induced deterioration of asthma is unknown.

Objectives: To compare bronchial inflammation and remodeling in never-smokers, ex-smokers, and current smokers with asthma.

Methods: A total of 147 patients with asthma (66 never-smokers, 46 ex-smokers, and 35 current smokers) were investigated.

Measurements and Main Results: Lung function, exhaled nitric oxide levels, and symptom questionnaires were assessed, and induced sputum and bronchial biopsies were obtained for determination of airway inflammation and remodeling. Smokers with asthma had lower FEV₁ and alveolar and bronchial nitric oxide levels than never-smokers. Smokers also had more goblet cells and mucus-positive epithelium, increased epithelial thickness, and a higher proliferation rate of intact and basal epithelium than ex-smokers and never-smokers. Smokers had higher numbers of mast cells and lower numbers of eosinophils than never-smokers. Ex-smokers had similar goblet cell numbers and mucus-positive epithelium, epithelial thickness, epithelial proliferation rate, and mast cell numbers as never-smokers.

Conclusions: Smokers with asthma have epithelial changes that are associated with increased asthma symptoms, such as shortness of breath and phlegm production. The fact that epithelial characteristics in ex-smokers are similar to those in never-smokers suggests that the smoke-induced changes can be reversed by smoking cessation.

AT A GLANCE COMMENTARY

SCIENTIFIC KNOWLEDGE ON THE SUBJECT

Smoking has detrimental effects on asthma outcome, such as increased cough, wheezing, sputum production, and frequency of asthma attacks. This ultimately results in accelerated lung function decline. The underlying pathological process of smoke-induced deterioration of asthma is unknown.

WHAT THIS STUDY ADDS TO THE FIELD

This study shows that smoking in asthma induces epithelial changes, thereby possibly increasing asthma symptoms such as shortness of breath and phlegm production. The fact that epithelial characteristics were similar in ex-smokers and never-smokers with asthma suggests that the smoke-induced changes can be reversed by smoking cessation.

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways characterized by variable airway obstruction and hyperresponsiveness. The clinical outcome of asthma is negatively affected by cigarette smoking, yet smoking is equally common in patients with asthma and the general population. Smokers with asthma have more severe asthma symptoms than nonsmokers with asthma (1), a higher frequency of asthma attacks (2), and accelerated lung function decline (3). The underlying pathological processes of smoke-induced deterioration of asthma are largely unknown.

Cigarette smoking has negative effects on the lung in subjects without asthma, associated with increased airway inflammation (e.g., increased neutrophil, macrophage, and CD8+ lymphocyte numbers in bronchoalveolar lavage fluid) (4–7) and increased inflammatory cell numbers in the bronchial wall, such as neutrophils, eosinophils, macrophages, and mast cells (5, 6, 8). Moreover, cigarette smoking has been associated with features of increased remodeling in subjects without asthma, such as increased tenascin and laminin deposition under the basement membrane (8).

Because smokers are often excluded in asthma studies to prevent coexistence of chronic obstructive pulmonary disease (COPD), only a few studies have been performed to investigate the effects of smoking on inflammation in asthma. These few studies were of small size and showed that cigarette smoking has an impact on asthmatic airway inflammation by increasing white blood cell numbers, sputum neutrophils (9, 10), and IL-8 levels (10) and by increasing the presence of bronchial metaplasia, neutrophil elastase and IFN- γ , and intraepithelial IL-8 (11) in the airway wall.

In the present study, we investigated the underlying pathological process of smoke-induced deterioration of asthma in a large asthma population. We hypothesized that smoking affects the outcome of asthma by increased bronchial inflammation and remodeling. To test this hypothesis, we included 147 patients with asthma (66 never-smokers, 46 ex-smokers, and 35 current smokers). Airway inflammation and remodeling were investigated in sputum samples and bronchial biopsies.

Some of the results of these studies have been previously reported in the form of an abstract (12).

METHODS

SUBJECTS

Smokers and nonsmokers with asthma ($n = 147$) were recruited (Table 1). All patients originated from cohorts investigated earlier by our research group, and all had a doctor's diagnosis of asthma and documented reversibility and bronchial hyperresponsiveness (BHR) to histamine in the past. Subjects were reexamined by lung function, BHR to histamine and/or adenosine 5'-monophosphate (AMP), sputum induction, and bronchoscopy with bronchial biopsies. Three groups were identified: (1) never-smokers (lifelong never-smokers; $n = 66$), (2) ex-smokers (had to have stopped smoking for at least 1 year and smoked at least 20 packs of cigarettes or 12 oz (360 g) of tobacco in a lifetime or at least one cigarette per day for 1 year or at least one cigar per week for 1 year [13]; $n = 46$), and (3) smokers (currently smoking; $n = 35$). Main exclusion criteria were FEV1 <1.2 L, bronchiectases, upper respiratory tract infection (e.g., colds), and/or use of antibiotics or oral corticosteroids within the last 2 months. The study protocol was approved by the local medical ethics committee. All subjects gave their written informed consent.

STUDY DESIGN

One or two weeks after blood eosinophil count, sputum induction and lung function assessments an AMP provocation test was performed, followed 1 to 2 weeks later by bronchoscopy. Subjects with a negative AMP provocation test (i.e., those with a

provocative concentration of AMP resulting in a 20% decline in FEV₁, AMP PC₂₀ >320 mg/ml) underwent a histamine provocation test more than 3 weeks later to determine BHR.

QUESTIONNAIRES

To determine cigarette smoke-induced symptoms, all patients were asked to fill out the clinical COPD questionnaire (CCQ) (14). Shortness of breath was determined by question 2: "On average, during the past week, how often did you feel short of breath doing physical activities?" (0 = never, 6 = almost all the time.) Sputum or phlegm production was determined by question 6: "In general, during the past week, how much of the time: did you produce phlegm?" (0 = never, 6 = almost all the time.)

LUNG FUNCTION

Lung function tests were performed with a daily calibrated spirometer according to standardized guidelines as described previously (15). FEV₁ was measured with a calibrated water-sealed spirometer according to standardized guidelines (15, 16). Reversibility of FEV₁ (% predicted) was measured after administration of 400 µg albuterol. Provocation tests were performed with a method adapted from Cockcroft and coworkers (17). After an initial nebulized 0.9% saline challenge, subjects inhaled doubling concentrations of AMP (0.04–320 mg/ml) by 2-minute tidal breathing at 5-minute intervals. BHR to histamine was tested as reported previously (18) using 30-second tidal breathing and doubling concentrations ranging from 0.13 to 32 mg/ml.

ALVEOLAR AND BRONCHIAL NITRIC OXIDE

Exhaled nitric oxide (NO) was measured on the Aerocrine NO system (Niox; Aerocrine AB, Stockholm, Sweden) in accordance with international guidelines (19). Alveolar NO concentrations (ppb) and bronchial NO fluxes (nl/s) were assessed according to Tsoukias and George (20) with some modifications (21).

ATOPY MEASUREMENTS

Atopy was determined by the Phadiatop screening test with the ImmunoCap system (Phadia AB, Uppsala, Sweden), and results are presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). Positive Phadiatop was defined as serum/control serum greater than 1.

SPUTUM INDUCTION AND PROCESSING

Sputum was induced by inhalation of 5% hypertonic saline aerosols over three consecutive periods of 5 minutes. Processed whole sputum samples were stained with May Grünwald Giemsa to obtain cell differentials by counting total 600 viable, nonsquamous cells. Sputum was not used if the percentage of squamous cells was greater than 80% or if the total number of nonsquamous cells was less than 600.

Sputum Supernatant Measurements

Histamine (IBL, Hamburg, Germany) and neutrophil elastase (Hycult, Uden, The Netherlands) were determined by ELISA. Eosinophilic cationic protein was measured using a fluoroenzyme immunoassay (ImmunoCAP ECP; Phadia AB).

COLLECTION, PROCESSING, IMMUNOHISTOCHEMICAL STAINING, AND CELLULAR QUANTIFICATION OF BRONCHIAL BIOPSIES

After local anesthesia, bronchial biopsies were obtained using a flexible bronchoscope (type Olympus BF P20 or BF XT20; Olympus, Center Valley, PA) from segmental divisions of the main bronchi. The biopsies were fixed in 4% formaldehyde solution, processed, and embedded in paraffin. Bronchial biopsies were cut into 3-µm-thick sections.

All histochemical stainings (periodic acid-shift [PAS] and H&E) were performed in 1 day using staining machines at the Pathology department. All immunohistochemical stainings (except for Ki67 and E-cadherin) were performed in an automated system

using the DAKO autostainer in three consecutive runs per cell marker. The slides were included in random fashion in each run to avoid groupwise staining. The inflammatory profile was assessed with specific antibodies against eosinophilic peroxidase (EPX) (laboratories of NA Lee and JJ Lee, Mayo Clinic, Scottsdale, AZ), mast cell tryptase (AA1; DAKO, Glostrup, Denmark), macrophages (CD68; DAKO), neutrophil elastase (NP57; DAKO), and T lymphocytes (CD3; DAKO). Sections were deparaffinized and, after antigen retrieval, incubated with the primary antibodies. These antibodies were detected with Envision Detection Kit (DAKO) followed by the chromogen NovaRED (Vector Labs, Burlingame, CA). EPX was detected using biotinylated antimouse IgG1 (Southern Biotech, Birmingham, AL) and alkaline phosphatase-labeled conjugate (DAKO) followed by permanent Red (DAKO). Sections were manually counterstained with methylgreen. Epithelial proliferation was assessed by Ki67 expression, epithelial adhesion by E-cadherin expression, and submucosal deposition of extracellular matrix by collagen 3 expression. Sections were deparaffinized and, after antigen retrieval, incubated with antibodies against Ki67 (DAKO), E-cadherin (BD Biosciences, Breda, the Netherlands), and collagen 3 (Southern Biotech). These antibodies were detected with two peroxidase-labeled conjugates (both DAKO). 3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used as chromogen, and hematoxylin (Ki67 and E-cadherin) or methylgreen (collagen 3) were used for counterstaining.

All stainings were quantified by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin; Leica Microsystems Imaging Solutions, Cambridge, UK). The largest of three biopsy sections was chosen for quantification. The number of positively stained inflammatory cells was counted in the submucosal area 100 μ m under the basement membrane (BM) in a total area of 0.1 mm² per biopsy sample. The number of goblet cells was counted on PAS-stained biopsy sections and expressed per mm of BM. The number of PAS-positive pixels was determined in the epithelium and expressed as the percentage of mucus-positive epithelium per biopsy section. Epithelial integrity was determined using H&E-stained biopsy sections and expressed as (1) percentage of BM covered with normal epithelium (a layer of basal and ciliated columnar epithelial cells without detachment from the BM) or (2) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells). The percentage of BM covered with E-cadherin-positive or E-cadherin-negative stained intact epithelium was determined. Epithelial thickness was determined by dividing the epithelial surface area by the BM length. Epithelial proliferation was determined by counting the number of Ki67-positive epithelial nuclei in intact and basal epithelium. Similarly to epithelial thickness, BM thickness was calculated by dividing the BM surface area by the BM length. Expression of collagen 3 was measured by computer-assisted image analysis in the submucosal area 200 μ m under the BM and was expressed as the percentage of positive tissue per biopsy section.

STATISTICS

All analyses were performed using SPSS (version 16.0; SPSS Inc., Chicago, IL). Normality of distributions was assessed using the Kolmogorov-Smirnov test. If necessary, normalization by transformation was attempted. Normally distributed data were analyzed with one-way analysis of variance and independent sample *t* tests. Nonnormally distributed data were analyzed with Kruskal-Wallis and Mann-Whitney *U* tests. Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson (for normally distributed data) or Spearman (for nonnormally distributed data: *rs*) tests. Multiple linear regression analysis was used to assess the influence of the independent variables inhaled corticosteroid (ICS) or long-acting β -agonist (LABA) use (yes/no) and smoking (smoking vs. never-smoking, smoking vs. ex-smoking, ex-smoking vs. never-smoking) on our different outcome variables (dependent variable) (see Tables E4 and E5 in the online supplement). Two-tailed *P* values < 0.05 were considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS

A total of 147 patients with asthma were included (66 never-smokers, 46 ex-smokers, and 35 current smokers). The number of pack-years was significantly lower in ex-smokers compared with smokers (Table 1). FEV₁%predicted (%pred) was significantly lower in ex-smokers and current smokers compared with never-smokers (Table 1). A higher FEV₁ (% pred) correlated significantly with a higher number of pack-years smoking in ex-smokers and current smokers (Spearman r [rs] = 0.22; P = 0.046). ICS use and dose were similar between never-smokers, ex-smokers, and current smokers (Table 1). The use of long-acting β -agonists (LABA) was significantly higher in ex-smokers compared with never-smokers and current smokers (Table 1). Alveolar and bronchial NO was significantly lower in smokers than in never-smokers and ex-smokers (Table 1). A lower alveolar and bronchial NO correlated with a higher number of pack-years in ex-smokers and current smokers (r s = -0.38; P = 0.004 and r s = -0.33; P = 0.014, respectively). A lower bronchial NO correlated with a higher number of cigarettes smoked daily in current smokers (r s = -0.58; P = 0.002). Self-reported symptoms, determined using the CCQ, were similar between the groups (Table E1).

SMOKE EXPOSURE AND MEDICATION USE

Because of the difference in pack-years between ex-smokers and current smokers, we tested within the ex-smoking group whether the number of pack-years affected our outcome variables. None of our outcome variables correlated significantly with the number of pack-years in the ex-smoking group (Table E3). Furthermore, we investigated the influence of ICS use and smoking on our data. Except for basement membrane (BM) thickness being lower with ICS use, none of the outcome variables was significantly influenced by ICS use (Table E4). The contribution of ICS use is visualized by indicating which patients are ICS users (open circles) and which are non-ICS users (solid circles) in the graphs in Figures 1 through 4. ICS and non-ICS users were randomly distributed. The influence of LABA use and smoking on our outcome variables was tested, and we found that LABA use did not significantly influence our data (Table E5).

BRONCHIAL EPITHELIAL REMODELING

A total of 114 patients with asthma underwent bronchoscopy successfully (49 never-smokers, 40 ex-smokers, and 25 current smokers). Smokers had higher numbers of goblet cells (Table 2) and a higher percentage of mucus-positive epithelium (Figures 1A and 1B; Table 2) compared with ex-smokers and never-smokers. They also had a thicker epithelial layer (Figures 1C and 1D; Table 2) and more proliferation of intact epithelium (Ki67+ intact epithelial cells; Figures 1E and 1F; Table 2). A similar increase in proliferation was observed in the basal epithelium of smokers compared with ex-smokers and never-smokers (Table 2). There were no significant differences in percentages of normal epithelium (Table 2) and metaplasia between the three groups or in the percentage of E-cadherin-positive intact epithelium, a marker of epithelial integrity (data not shown). In ex-smokers and current smokers, proliferation of intact and basal epithelial cells was significantly higher with an increasing number of pack-years (r s = 0.28; P = 0.026 and r s = 0.33; P = 0.007, respectively). Epithelial thickness correlated with the percentage of mucus-positive epithelium (Figure 2A) and the percentage of Ki67+ intact (Figure 2B) and basal epithelial cells (r s = 0.29; P = 0.009). There were no significant correlations between lung function parameters (e.g., FEV₁) and all epithelial parameters.

TABLE 1: PATIENT CHARACTERISTICS

	Never-smokers N=66	Ex-smokers N=46	Smokers N=35
Sex (m/f)	28/38	27/19	19/16
Age (yr)	47 (19-71)	52 (25-68) [§]	50 (21-64) [*]
Packyears (yr)	-	3 (0-64) [§]	15 (0.4-47) ^{*,#}
Cigarettes / day (n)	-	-	14 (3-25) ^{*,#}
ICS use (yes/no)	27/39	25/21	12/23
ICS dose (µg/day) beclomethasone equivalent	1000 (100-2000)	800 (28-2000)	650 (200-1500)
Long-Acting β ₂ -agonist use n (%)	17 (26)	21 (46) [§]	8 (23) [*]
Atopy n (%)	49 (74)	29 (63)	22 (64)
FEV ₁ (L)	3.3 (2.2-6.3)	3.2 (1.5-5.8)	3.1 (1.8-5.1)
FEV ₁ (% pred)	104 (58-135)	94 (43-123) [§]	98 (60-119) [#]
FEV ₁ /VC (%)	77 (55-98)	72 (39-91) [§]	75 (48-94)
Reversibility (% pred)	8.8 (-1.4-38.4)	7.7 (-8.4-28.4)	10 (-2.2-33.2)
MEF ₅₀ (% pred)	74 (24-152)	65 (17-147) [§]	70 (26-124)
PC ₂₀ AMP (mg/ml)	62.5 (0-640)	115.5 (0.08-640)	28.4 (0.02-640)
NO alveolar (ppb)	6.3 (3.1-51.7)	5.6 (1.5-14.9)	4.3 (2.1-10.4) ^{*,#}
NO bronchial (nL/s)	1.02 (0.12-10.4)	0.93 (0.22-4.1)	0.38 (0.06-1.4) ^{*,#}
Blood eosinophils (x10 ⁹ /L)	0.17 (0-4.6)	0.21 (0-0.78)	0.23 (0-0.49)

Values are medians (ranges). Packyears = smoking history, cigarettes per day = current smoking. ICS (inhaled corticosteroid) dose only in subjects using ICS and expressed as the dose of beclomethasone, all other ICS doses were converted to their equivalent doses of beclomethasone. Atopy was based on a positive Phadiatop test; specific IgE's in patient serum/control serum >1. FEV₁= forced expiratory volume in 1 s, FEV₁ was measured after inhalation of 800 µg Albuterol. VC= vital capacity. Reversibility FEV₁ = change in FEV₁, expressed as increase in percentage predicted normal value after 400 µg of Albuterol; MEF₅₀= maximum expiratory flow rate at 50% of vital capacity. PC₂₀ AMP = provocative concentration of adenosine 5'monophosphate causing a 20% fall in FEV₁ n= number.

^{*} = smokers vs. ex-smokers p<0.05

[#] = smokers vs. never-smokers p<0.05

[§] = ex-smokers vs. never-smokers p<0.05

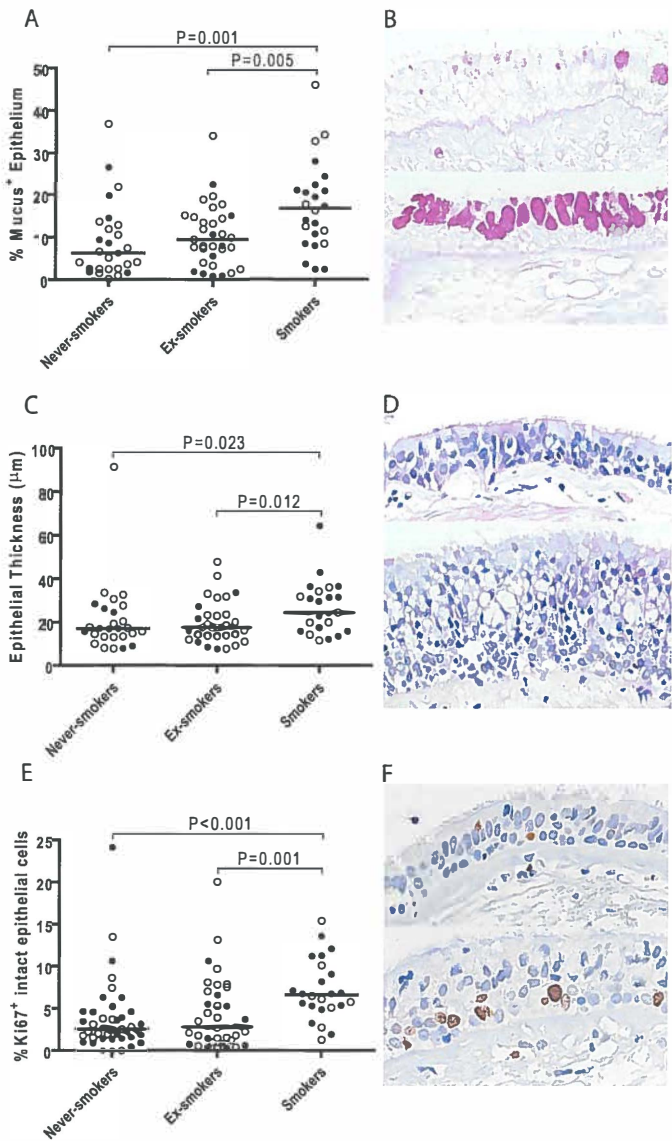


Figure 1
Epithelial morphology. (A) Percentage of mucus-positive epithelium in asthmatic never-, ex- and current smokers. (B) Pictures of PAS stained goblet cells in never- (upper picture) and current smokers (lower picture). (C) Epithelial thickness in asthmatic never-, ex- and current smokers. (D) Pictures of epithelial thickness in never- (upper picture) and current smokers (lower picture). (E) Percentage of Ki67+ intact epithelial cells in asthmatic never-, ex- and current smokers. (F) Pictures of Ki67 expression in intact epithelium in never- (upper picture) and current smokers (lower picture). Lens magnification 400x. Each circle represents one subject; closed circles are non-ICS users and open circles are the ICS users. Horizontal bars represent median values.

TABLE 2: REMODELING IN BRONCHIAL BIOPSIES

		Never-smokers N=49	Ex-smokers N=40	Smokers N=25
Epithelium	Goblet cells (N)	31 (4-220)	36 (2-69)	65 (21-119)* [#]
	Mucus (%)	6.2 (0.2-37.0)	9.4 (0.8-33.9)	16.9 (2.5-46.1)* [#]
	Thickness (μm)	17.0 (7.9-91.4)	17.8 (7.5-48.1)	24.6 (11.8-64.4)* [#]
	Ki67 ⁺ (%intact)	2.6 (0-24.2)	2.8 (0.2-20.1)	6.6 (1.3-15.5)* [#]
	Ki67 ⁺ (%basal)	2.6 (0-10.7)	2.7 (0-28.9)	5.6 (1.2-25.5)* [#]
	Normal epithelium (%)	7.6 (0-65.3)	9.7 (0-50.7)	9.5 (0-32.8)
Basement Membrane	Thickness (μm)	6.1 (3.1-11.7)	5.7 (2.8-12.6)	6.4 (3.7-9)

Values are medians (ranges). Number of goblet cells is expressed per mm basement membrane. Mucus is expressed as the percentage of mucus-positive epithelium. Ki67 expression is presented as the percentage of Ki67-positive nuclei of all epithelial cell nuclei in either intact or basal epithelium. Normal epithelium is the percentage of the BM covered with normal epithelium.

* = smokers vs. ex-smokers $p < 0.05$

= smokers vs. never-smokers $p < 0.05$

[‡] = ex-smokers vs. never-smokers $p < 0.05$

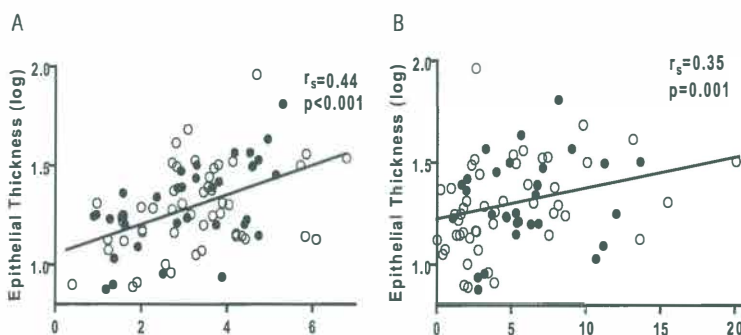


Figure 2

Correlations with epithelial thickness. (A) Correlation between the percentage of mucus-positive epithelium (sqrt transformed) and epithelial thickness (log transformed) in asthmatic never, ex- and current smokers. (B) Correlation between the percentage of Ki67+ intact epithelial cells and epithelial thickness (log transformed) in asthmatic never, ex- and current smokers. Each circle represents one subject; closed circles are non-ICS users and open circles are the ICS users.

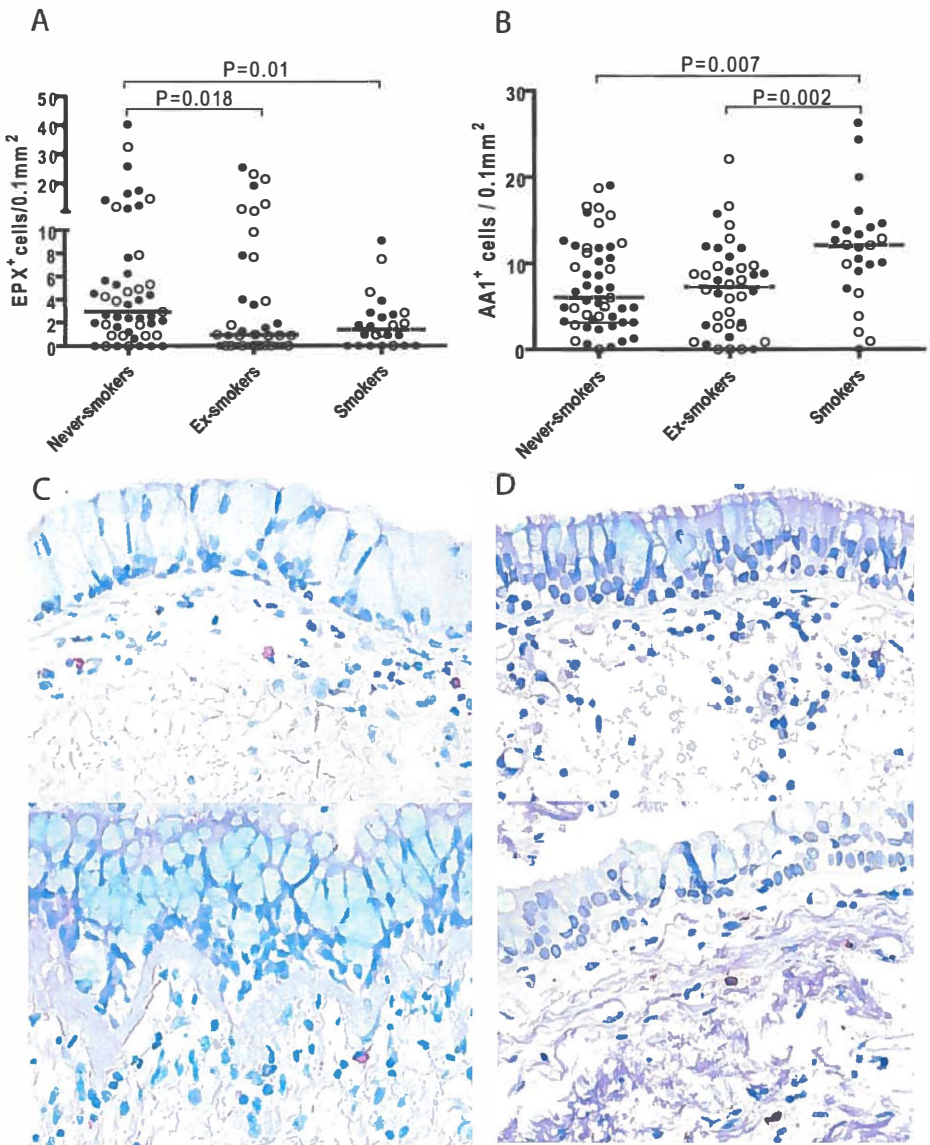


Figure 3
Eosinophils and mast cells in bronchial biopsies. (A) The number of EPX⁺ eosinophils in 0.1 mm² bronchial biopsy tissue. (B) The number of AA1⁺ mast cells in 0.1 mm² bronchial biopsy tissue. Representative pictures of (C) EPX⁺ eosinophils in biopsies of asthmatic never-smokers (upper picture) and current smokers (lower picture) and (D) AA1⁺ mast cells in biopsies of asthmatic never-smokers (upper picture) and current smokers (lower picture). Lens magnification 400x. Each circle represents one subject; closed circles are non-ICS users and open circles are the ICS users. Horizontal bars represent median values.

CORRELATIONS BETWEEN BRONCHIAL EPITHELIAL REMODELING AND SELF-REPORTED SYMPTOMS

Analysis of the complete study population revealed that the severity of self-reported phlegm production (CCQ, question 6) correlated significantly with the number of goblet cells ($r_s = 0.26$; $P = 0.034$), presence of mucus-positive epithelium ($r_s = 0.23$; $P = 0.045$), and epithelial thickness ($r_s = 0.29$; $P = 0.011$). The severity of self-reported shortness of breath (CCQ, question 2) correlated significantly with epithelial thickness ($r_s = 0.26$; $P = 0.018$).

OTHER PARAMETERS OF AIRWAY REMODELING

BM thickness (Table 2) and collagen 3 deposition (not shown) were similar between never-smokers, ex-smokers and current smokers.

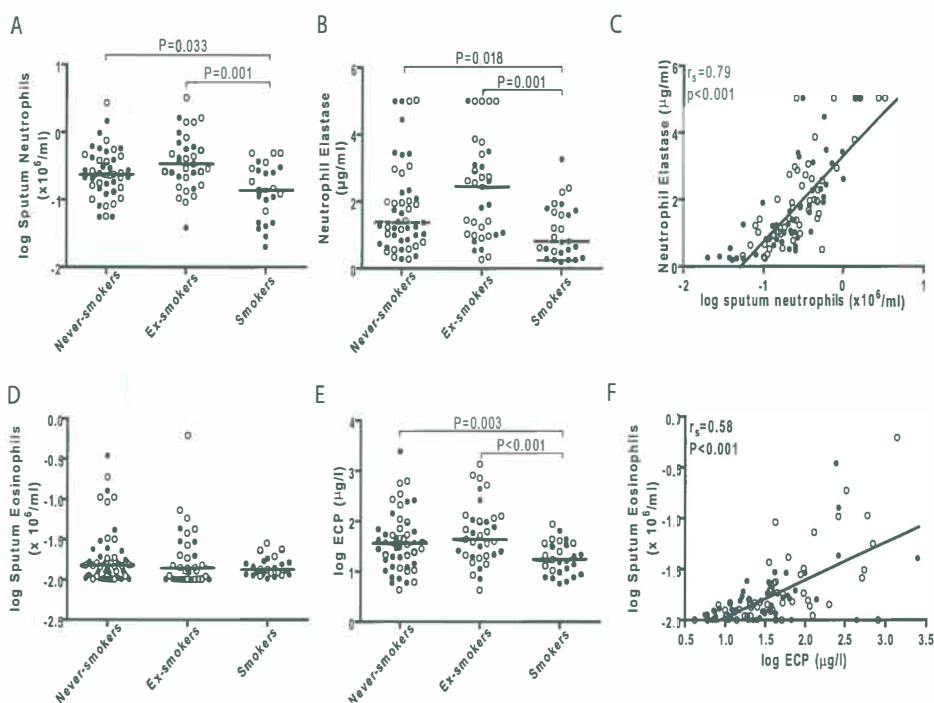


Figure 4. Neutrophilic and eosinophilic inflammation in sputum. (A) The absolute number of sputum neutrophils (log transformed) in asthmatic never-, ex- and current smokers. (B) Sputum concentration of neutrophil elastase in asthmatic never-, ex- and current smokers. (C) Correlation between the absolute number of sputum neutrophils (log transformed) and the sputum concentration of neutrophil elastase. (D) The absolute number of sputum eosinophils (log transformed) in asthmatic never-, ex- and current smokers. (E) Sputum concentration of eosinophilic cationic protein (ECP, log transformed) in asthmatic never-, ex- and current smokers. (F) Correlation between the absolute number of sputum eosinophils (log transformed) and the sputum concentration of eosinophilic cationic protein (ECP, log transformed). Each circle represents one subject; closed circles are non-ICS users and open circles are the ICS users. Horizontal bars represent median values.

INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

Smokers and ex-smokers had lower numbers of EPX⁺ eosinophils than never-smokers (Figures 3A and 3C). The number of EPX⁺ eosinophils in biopsies increased with increasing duration of smoking cessation in ex-smokers ($r_s = 0.32$; $P = 0.044$). Smokers had a higher number of bronchial AA1⁺ mast cells than ex-smokers and never-smokers (Figures 3B and 3D). Bronchial EPX⁺ eosinophils or AA1⁺ mast cells did not significantly correlate with lung function, symptoms, or epithelial remodeling. The number of airway wall neutrophils, macrophages, and CD3-positive lymphocytes did not differ significantly between the three groups (Table 3).

INFLAMMATORY CELLS AND MEDIATORS IN SPUTUM

A total of 105 patients with asthma underwent sputum induction successfully (47 never-smokers, 33 ex-smokers, and 25 current smokers). The percentage and absolute number of sputum macrophages was highest in never-smokers. The absolute number of sputum neutrophils was slightly but significantly increased in never-smokers and ex-smokers compared with current smokers (Figure 4A; Table E2). Similar to the percentage and absolute number of neutrophils, the level of neutrophil elastase in sputum was highest in ex-smokers (Figure 4B; Table E2). The level of neutrophil elastase correlated significantly with the number of sputum neutrophils (Figure 4C). Nine patients had neutrophil elastase levels that were above the measurement threshold, and the level of these patients was set at a maximum value of 5. When these patients were excluded, a significant correlation was still present between neutrophil elastase and the number of sputum neutrophils.

Sputum eosinophils did not differ significantly between the three groups (Figure 4D; Table E2). However, the level of eosinophilic cationic protein was significantly lower in smokers compared with ex-smokers and never-smokers (Figure 4E) and correlated significantly with the number of sputum eosinophils (Figure 4F). The percentage and absolute numbers of sputum lymphocytes were lower in ex-smokers and current smokers compared with never-smokers. The level of histamine in sputum supernatant was similar between the three groups (Table E2).

TABLE 3: INFLAMMATORY CELLS IN BRONCHIAL BIOPSIES

Cells/0.1mm ²	Never-smokers N=49	Ex-smokers N=40	Smokers N=25
EPX ⁺ eosinophils	3 (0-40)	1 (0-26) ^{\$}	1 (0-9) [#]
AA1 ⁺ mast cells	6 (0-19)	7 (0-22)	12 (0-26) ^{*,#}
CD68 ⁺ macrophages	15 (3-36)	11 (0-57)	10 (0-37)
NP57 ⁺ neutrophils	6 (0-46)	9 (0-34)	6 (0-20)
CD3 ⁺ lymphocytes	77 (21-294)	67 (4-219)	62 (13-216)

Values are medians (ranges).

* = smokers vs. ex-smokers $p < 0.05$

= smokers vs. never-smokers $p < 0.05$

\$ = ex-smokers vs. never-smokers $p < 0.05$.

DISCUSSION

This is the first study in a large population of patients with asthma to compare current, ex-smokers, and never-smokers. The results show that cigarette smoking increases bronchial epithelial remodeling and thereby possibly asthma symptoms, such as shortness of breath and phlegm production. Concerning inflammation, smoking in asthma was characterized by lower bronchial eosinophil and higher mast cell numbers. Because the smoking-related changes in epithelium and mast cell numbers were not observed in ex-smokers, this suggests that these smoking-related changes are reversible by smoking cessation.

Smoking associated with increased epithelial cell proliferation in our asthma population, and this increase correlated with a higher number of pack-years. It has been reported that cigarette smoke induces epithelial regeneration to restore the bronchial epithelium after smoke-induced damage (22) by epithelial cell migration, proliferation, and differentiation (23). Chronic exposure to cigarette smoke can disturb adequate epithelial regeneration, leading to excessive epithelial proliferation (24, 25) and squamous or mucous cell metaplasia (22). This excessive epithelial proliferation can lead to thickening of the epithelial layer, as we observed in our smokers with asthma and as was previously shown in smokers without asthma (26). The epithelial thickening was associated with increased proliferation of intact and basal epithelial cells and by goblet cell hyperplasia. Goblet cell hyperplasia and increased mucus production may be caused directly by cigarette smoke exposure (27) or indirectly via the activation of mucin gene transcription by inflammatory cell mediators (22).

Our data suggest that the increased epithelial thickness may underlie increased respiratory symptoms. This is supported by the correlation between epithelial thickness and self-reported shortness of breath and the correlations between epithelial thickness, goblet cell numbers, and mucus-positive epithelium with self-reported phlegm production. Therefore, it can be envisaged that cigarette smoking causes an increase in bronchial epithelial thickness in the asthmatic lung by epithelial hyperplasia, contributing to narrowing of the airway and resulting in increased shortness of breath and, indirectly via goblet cell hyperplasia, increased phlegm production. Over the longer term, this might contribute to the decreased lung function as shown by significantly lower FEV₁%predicted values in smokers with asthma compared with never-smokers.

In asthma, exhaled NO has been put forward as a reliable indicator of disease activity (28). We show, in line with a previous report (29), that exhaled nitric oxide (NO) is lower in smokers than in nonsmokers with asthma. Moreover, in ex-smokers and current smokers with asthma, a lower level of alveolar and bronchial NO was associated with a higher number of pack-years. Several mechanisms for a reduction in NO by cigarette smoke have been suggested, such as smoke-induced down-regulation of NO synthase by high concentrations of NO in cigarette smoke (30) and inactivation of NO by oxidants present in cigarette smoke or by smoke-induced toxic damage to NO-producing cells (31). The observed suppressive effect of cigarette smoke on exhaled NO negatively affects its value as a reliable marker to monitor disease activity in smokers with asthma.

The inflammatory profile appeared to be different in smokers compared with ex-smokers and never-smokers with asthma, as shown by higher numbers of mast cells and lower numbers of eosinophils in the bronchial airway wall. Thus, cigarette smoking in asthma suppressed eosinophilic inflammation, a characteristic of asthma, whereas it did not change the inflammatory pattern toward a COPD-like profile, which characteristically would be dominated by macrophages and neutrophils in sputum and bronchoalveolar lavage fluid, and T lymphocytes and macrophages in bronchial biopsies (32).

The increase in mast cell numbers as observed in smokers with asthma was in line with reports showing increased mast cell numbers in lungs and skin of asymptomatic smokers (33). Mast cells are thought to promote smoke-induced airway remodeling (34) and mucus hypersecretion (35) and were shown in an experimental mouse model

to contribute to the initiation and maintenance of airway epithelial proliferation after chronic exposure to ozone (36). In our study, we did not find a correlation between mast cell numbers and epithelial changes (i.e., epithelial layer thickness, goblet cells, and proliferation). Therefore, our data do not support a mast cell-driven mechanism to explain bronchial epithelial changes and deteriorating asthma symptoms in smokers with asthma.

Eosinophil numbers were higher in never-smokers than in smokers and ex-smokers in our study. Numbers of EPX+ eosinophils increased with increasing duration of smoking cessation in ex-smokers, suggesting that smoking cessation reverses the down-regulatory effect of smoking on the number of eosinophils. These data indicate that smoking inhibits asthma-related eosinophilia not only acutely but also as a sustained effect that only gradually decreases after smoking cessation. Smoke-related decreases in eosinophilic inflammation in asthma have also been reported by others in blood (37) and sputum (10). In addition, we and others showed a down-regulatory effect of smoking on ovalbumin-induced airway eosinophilia in a mouse model of asthma (38, 39). Mechanisms involved in the reduction of eosinophilic airway inflammation by smoking in asthma may include reduced eosinophil recruitment from the bone marrow or increased apoptosis of activated eosinophils by the actions of exogenous NO (40) or carbon monoxide (41) in cigarette smoke.

With respect to neutrophils, ex-smokers with asthma had higher neutrophil numbers and neutrophil elastase levels in sputum than their smoking counterparts. An increase in sputum neutrophil numbers has also been reported in ex-smokers with COPD (42) and is possibly caused by the reduction of antiinflammatory factors after smoking cessation (e.g., CO) that are present in cigarette smoke. Although neutrophil infiltration may contribute to goblet cell degranulation (43) and lung tissue destruction (44), we did not observe these changes in airways of ex-smokers with asthma. Neutrophils also play a role in normal tissue repair and thus in restoration of tissue homeostasis after taking away the smoking-induced tissue-damage effect (42).

Tsoumakidou and colleagues recently found that cigarette smoking in subjects with asthma caused a reduction in mucosal CD83+ mature dendritic cells and CD20+ B cells compared with never-smokers with asthma and healthy control subjects (45). With respect to our results, it can be envisaged that such a loss of dendritic cells might contribute to an alteration in the balance of inflammatory cell phenotypes, concurrent with lower bronchial eosinophil and higher mast cell numbers as was observed in the smokers with asthma in our study. This is compatible with the suggestion that a change in airway immunity may be responsible for the increases in exacerbation frequency and the accelerated lung function decline observed in smokers with asthma (46).

We studied a population of subjects with asthma that was heterogeneous not only in smoking habits and history but also in medication use. Therefore, ICS or LABA use together with smoking might have influenced our results. Because medication use did not significantly influence our outcome variables, we can rule out the possibility that this caused the differences in airway remodeling and inflammation between the never-smokers, ex-smokers, and current smokers. However, one of our outcome variables, BM thickness, was reduced by ICS use rather than by smoking. A thickened BM is a specific characteristic of airway remodeling in asthma, and, although still controversial, ICS treatment has been shown in several studies to reduce BM thickness (47–50), which is supported by our data.

The inclusion of ex-smokers with asthma in our study enabled us to investigate the possible reversibility of smoke-related changes upon smoking cessation. Our ex-smoking group had a significantly lower number of pack-years compared with the smoking group, suggesting that differences between these two groups might be due to lower cumulative smoke exposure in ex-smokers. This was not the case because the number of pack-years was not significantly correlated to any of our outcome variables in the ex-smoking group. It is possible that current rather than past exposure to cigarette smoke is important for the bronchial changes we observed. With respect to bronchial remodeling and inflammation, ex-smokers with asthma differed from smokers with

asthma with significantly less epithelial layer thickness, mucus-producing epithelium, epithelial cell proliferation, and airway wall mast cell numbers. Although epithelial changes were associated with higher self-reported asthma symptoms in smokers, lung function and self-reported asthma symptoms were not significantly different between ex-smokers and current smokers. This indicates that epithelial thickness does not solely cause the symptomatology and severity of airway obstruction in smokers with asthma but may be an important contributor. Another important aspect when interpreting our study is its cross-sectional design, necessitating confirmation in a longitudinal study on smoking cessation in asthma. Nevertheless, based on our observations, we speculate that the smoke-related epithelial changes and increase in mast cell numbers are reversible by smoking cessation.

In conclusion, we show in a large population of patients with asthma that cigarette smoking induces epithelial changes in association with increases in asthma symptoms, such as shortness of breath and phlegm production. Cigarette smoking in asthma was associated with lower bronchial eosinophil and higher mast cell numbers. The fact that epithelial changes and higher mast cell numbers were not observed in ex-smokers with asthma suggests that these smoke-induced changes can be reversed by smoking cessation.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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Airway epithelial changes in smoking but not in ex-smoking asthmatics

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Online data supplement

A human custom multiplex antibody bead kit (Biosource, Invitrogen Ltd, Paisley, UK) was used to measure IFN- γ , IL-4, 5, 8, 13, 17, EGF, Eotaxin, FGFB, GM-CSF, MCP1, RANTES, VEGF by Luminex, according to the manufacturers' instructions. Data was analysed using Starstation software. For each cytokine or growth factor undetectable values, those below the detection limit, were set at 0 (detection limits: IFN- γ : 5, IL-4: 5, IL-5: 3, IL-8: 3, IL-13: 10, IL-17: 10, EGF: 25, Eotaxin: 5, FGFB: 20, GM-CSF: 15, MCP1: 10, RANTES: 15, VEGF: 15 pg/ml).

We detected no significant differences in the level of the inflammatory cytokines and growth factors IFN- γ , IL-4, 5, 8, 13, 17, EGF, Eotaxin, FGFB, GM-CSF, MCP1, RANTES and VEGF between subjects with asthma remission and asthma patients with a slow or a fast FEV1 decline.

TABLE E1: Clinical COPD questionnaire (CCQ) data

	Never-smokers N=47	Ex-smokers N=33	Current smokers N=31
On average during the past week, how often did you feel:			
q.1 Short of breath at rest?	0 (0-3)	0 (0-3)	0 (0-2)
q.2 Short of breath doing physical activities?	2 (0-4)	1 (0-6)	1 (0-5)
q.3 Concerned about getting a cold or your breathing getting worse?	0 (0-1)	0 (0-3)	0 (0-2)
q.4 Depressed (down) because of your breathing problems?	0 (0-2)	0 (0-3)	0 (0-2)
In general, during the past week, how much of the time:			
q.5 Did you cough?	1 (0-6)	2 (0-4)	2 (0-4)
q.6 Did you produce phlegm?	1 (0-4)	2 (0-6)	2 (0-4)
On average, during the past week, how limited were you in these activities because of your breathing problems:			
q.7 Strenuous physical activities (such as climbing stairs, hurrying, doing sports)?	1 (0-5)	1 (0-6)	1 (0-4)
q.8 Moderate physical activities (such as walking, housework, carrying things)?	0 (0-3)	0 (0-5)	0 (0-3)
q.9 Daily activities at home (such as dressing, washing yourself)?	0 (0-2)	0 (0-3)	0 (0-2)
q.10 Social activities (such as talking, being with children, visiting friends/relatives)?	0 (0-2)	0 (0-2)	0 (0-3)
CCQ Total score	8 (0-18)	7 (0-30)	6.5 (0-24)
Total domain score symptoms	5 (0-11)	5.5 (0-16)	5.5 (0-11)
Total domain score functional	2 (0-10)	1 (0-12)	1 (0-11)
Total domain score mental	0 (0-3)	0 (0-6)*	0 (0-3)

Values are medians (ranges). Patients were asked to check the number of the response that best described how they have been feeling during the past week. For questions 1-6 patients could chose from the answers: 0=never, 1=hardly ever, 2=a few times, 3=several times, 4=many times, 5=a great many times, 6=almost all the time. For questions 7-10 patients could chose from the answers: 0=not limited at all, 1=very slightly limited, 2=slightly limited, 3=moderately limited, 4=very limited, 5=extremely limited, 6=totally limited/or unable to do.

* P<0.05 vs. never-smokers.

TABLE E2: INFLAMMATORY CELLS AND MEDIATORS IN SPUTUM

	Never-smokers N=47	Ex-smokers N=33	Smokers N=25
Total cells ($\times 10^6$ /ml)	0.50 (0.03-3.00)	0.50 (0.06-3.86)	0.32 (0.05-1.05)*, #
Macrophages (%)	42.0 (4.9-82.5)	26.9 (5.3-74.7)\$	38.1 (7.1-76.5)*
($\times 10^6$ /ml)	0.15 (0.03-1.07)	0.15 (0.01-0.61)	0.10 (0.02-0.61)*, #
Neutrophils (%)	47.4 (15.6-94)	67.6 (22.5-94.5)\$	55.8 (21.6-92.7)*
($\times 10^6$ /ml)	0.22 (0.05-2.70)	0.32 (0.03-3.23)	0.12 (0.01-0.48)*, #
Eosinophils (%)	0.8 (0-67.1)	0.5 (0-38.6)	1.4 (0.2-5.9)
($\times 10^6$ /ml)	0.005 (0-0.34)	0.004 (0-0.61)	0.003 (0-0.02)
Lymphocytes (%)	0.9 (0-6.9)	0.3 (0-3.3)\$	0.2 (0-1.1)#
($\times 10^6$ /ml)	0.004 (0-0.11)	0.002 (0-0.07)\$	0.0006 (0-0.008)#
Neutrophil Elastase (μ g/ml)	1.4 (0.2-5)	2.4 (0.3-5)	0.8 (0.2-3.3)*, #
Eosinophilic Cationic Protein (ECP) (μ g/l)	35.0 (4-2467)	42.7 (4-1363)	17.4 (6-88)*, #
Histamine (ng/ml)	27.8 (0.2-129.5)	28.8 (5.6-134.1)	25.6 (0-215)

Values are medians (ranges).

* = smokers vs. ex-smokers $p < 0.05$

= smokers vs. never-smokers $p < 0.05$

\$ = ex-smokers vs. never smokers $p < 0.05$.

TABLE E3: CORRELATION WITH SMOKE EXPOSURE-RELATED VARIABLES IN EX-SMOKERS

	Packyears		Duration of smoking cessation	
	<i>r_s</i>	p value	<i>r_s</i>	p value
FEV ₁ (%pred)	-0.096	0.53	-0.184	0.23
NO alveolar (ppb)	-0.223	0.24	0.332	0.07
NO bronchial (nL/s)	-0.105	0.58	0.212	0.26
Goblet cells (N)	-0.283	0.12	0.182	0.32
Mucus-positive epithelium (%)	-0.028	0.88	0.040	0.83
Epithelial thickness (µm)	0.202	0.26	0.126	0.49
Ki67 ⁺ intact epithelium (%)	-0.033	0.84	-0.076	0.64
Ki67 ⁺ basal epithelium (%)	0.176	0.29	0.131	0.43
Eosinophils (per 0.1mm ² tissue)	0.161	0.32	-0.023	0.89
Mast cells (per 0.1mm ² tissue)	-0.232	0.15	0.320	0.04*

*P<0.05, the table only shows those variables that were significantly different in smoking asthmatics compared to ex- and never-smokers

TABLE E4: Multiple linear regression analyses – ICS use and smoking

Dependent Variable	R square	ICS use	ICS use	S vs. NS	S vs. NS
		(y/n) Beta	(y/n) p value	Beta	p value
FEV ₁ (%pred)	0.077	-0.147	0.136	-0.244	0.014*
NO alveolar (ppb)	0.187	0.119	0.295	-0.408	0.001*
NO bronchial (nL/s)	0.230	0.138	0.212	-0.450	<0.001*
Goblet cells (N)	0.138	0.089	0.495	0.366	0.007*
Mucus-positive epithelium (%)	0.199	-0.019	0.884	0.444	0.001*
Epithelial thickness (μm)	0.108	-0.081	0.555	0.314	0.026*
Mast cells (per 0.1mm ² tissue)	0.086	-0.057	0.620	0.283	0.015*
Basement membrane thickness (μm)	0.130	-0.357	0.002*	0.038	0.737
	R square	ICS use	ICS use	S vs. EX	S vs. EX
		(y/n) Beta	(y/n) p value	Beta	p value
FEV ₁ (%pred)	0.018	-0.125	0.280	0.028	0.806
NO alveolar (ppb)	0.100	0.088	0.509	-0.287	0.035*
NO bronchial (nL/s)	0.264	0.069	0.570	-0.496	<0.001*
Goblet cells (N)	0.306	0.178	0.173	0.560	<0.001*
Mucus-positive epithelium (%)	0.133	0.026	0.842	0.369	0.006*
Epithelial thickness (μm)	0.111	-0.037	0.779	0.324	0.016*
Mast cells (per 0.1mm ² tissue)	0.106	-0.011	0.931	0.323	0.011*
Basement membrane thickness (μm)	0.145	-0.333	0.007*	0.130	0.282
	R square	ICS use	ICS use	EX vs. NS	EX vs. NS
		(y/n) Beta	(y/n) p value	Beta	p value
FEV ₁ (%pred)	0.080	-0.098	0.299	-0.253	0.008*
NO alveolar (ppb)	0.047	0.167	0.166	-0.164	0.175
NO bronchial (nL/s)	0.030	0.173	0.156	-0.039	0.746
Goblet cells (N)	0.013	0.079	0.520	-0.097	0.430
Mucus-positive epithelium (%)	0.024	-0.068	0.611	0.149	0.265
Epithelial thickness (μm)	0.011	-0.104	0.435	0.021	0.872
Mast cells (per 0.1mm ² tissue)	0.009	-0.068	0.534	-0.056	0.608
Basement membrane thickness (μm)	0.136	-0.348	0.001*	-0.086	0.401

* p<0.05, the table only shows those variables that were significantly different in smoking asthmatics compared to ex- and never-smokers (except for basement membrane thickness) and were normally distributed (if necessary after transformation). ICS = inhaled corticosteroids, S = smoking, NS = never smoking, EX = ex-smoking.

TABLE E5: Multiple linear regression analyses - LABA use and smoking

Dependent Variable	R square	LABA use (y/n) Beta	LABA use (y/n) p value	S vs. NS Beta	S vs. NS p value
FEV ₁ (%pred)	0.076	-0.145	0.141	-0.240	0.016*
NO alveolar (ppb)	0.176	0.055	0.632	-0.414	0.001*
NO bronchial (nL/s)	0.214	0.056	0.616	-0.458	<0.001*
Goblet cells (N)	0.130	-0.020	0.877	0.360	0.008*
Mucus-positive epithelium (%)	0.205	-0.083	0.524	0.443	0.001*
Epithelial thickness (µm)	0.102	-0.017	0.904	0.318	0.024*
Mast cells (per 0.1mm ² tissue)	0.087	-0.068	0.549	0.285	0.014*
	R square	LABA use (y/n) Beta	LABA use (y/n) p value	S vs. EX Beta	S vs. EX p value
FEV ₁ (%pred)	0.019	-0.132	0.259	0.022	0.848
NO alveolar (ppb)	0.093	-0.004	0.975	-0.306	0.027*
NO bronchial (nL/s)	0.262	-0.052	0.668	-0.522	<0.001*
Goblet cells (N)	0.284	0.097	0.463	0.548	<0.001*
Mucus-positive epithelium (%)	0.133	-0.012	0.929	0.361	0.008*
Epithelial thickness (µm)	0.113	0.054	0.682	0.344	0.012*
Mast cells (per 0.1mm ² tissue)	0.105	0.000	0.994	0.324	0.011*
	R square	LABA use (y/n) Beta	LABA use (y/n) p value	EX vs. NS Beta	EX vs. NS p value
FEV ₁ (%pred)	0.078	-0.086	0.366	-0.248	0.010*
NO alveolar (ppb)	0.030	0.101	0.409	-0.162	0.188
NO bronchial (nL/s)	0.006	0.077	0.534	-0.032	0.795
Goblet cells (N)	0.008	-0.015	0.906	-0.083	0.503
Mucus-positive epithelium (%)	0.036	-0.131	0.328	0.167	0.214
Epithelial thickness (µm)	0.001	-0.030	0.827	0.014	0.920
Mast cells (per 0.1mm ² tissue)	0.008	-0.067	0.544	-0.051	0.644

* p<0.05, the table only shows those variables that were significantly different in smoking asthmatics compared to ex- and never-smokers and were normally distributed (if necessary after transformation). LABA = long-acting beta-agonists, S = smoking, NS = never smoking, EX = ex-smoking.

Clinical Control of Asthma Associates with Measures of Airway Inflammation

Submitted

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ABSTRACT

Background: Control of asthma is the goal of asthma management worldwide. The Global Initiative for Asthma (GINA, 2006) defined control by a composite measure of clinical findings but without using markers of airway inflammation, the hallmark of asthma. We investigated whether such markers and quality of life are associated with the level of asthma control.

Methods: Control of asthma was assessed over a period of four weeks in 111 asthmatics: 22 totally controlled, 47 well controlled, and 42 uncontrolled. Lung function, quality of life, airway hyperresponsiveness to adenosine-5'-monophosphate, sputum and blood eosinophils, exhaled nitric oxide and bronchial biopsies were obtained.

Findings: The 69 subjects with controlled asthma (totally and well controlled combined) had lower median blood eosinophil numbers, slope of hyperresponsiveness to adenosine-5'-monophosphate, and alveolar nitric oxide levels than the 42 subjects with uncontrolled asthma: 0.18 (range 0.01-0.54) versus 0.22 (0.06-1.16) $\times 10^9/L$ ($p < 0.05$), 3.8 (-0.4-17750) versus 39.7 (0.4-28000) mg/mL ($p < 0.05$), and 5.3 (1.5-14.9) versus 6.7 (2.6-51.7) ppb ($p < 0.05$) respectively. Biopsies from subjects with controlled asthma contained fewer eosinophilic granules (113 (6-1787) versus 219 (19-5313) ($p < 0.05$) and more intact epithelium (11.8 (0-65.3) versus 5.6 (0-47.6)% ($p < 0.05$) than uncontrolled subjects. Controlled asthmatics had better Asthma Quality of Life Questionnaire scores than uncontrolled patients: 6.7 (5.0-7.0) versus 5.9 (3.7-7.0) ($p < 0.001$).

Interpretation: The level of control of asthma, based on a composite measure of clinical findings, is associated with both direct and indirect markers of inflammation and is related to patients' quality of life.

INTRODUCTION

Aiming for total control of asthma by applying strict rules for treatment was the focus of the Gaining Optimal Asthma control (GOAL) study in 2004(1). In this landmark study, totally controlled and well controlled asthma were composite measures based on respiratory symptoms during the day and night, use of rescue medication, peak expiratory flow rate, exacerbations, emergency visits to the doctor or hospital, and adverse side-effects of treatment(1). Indeed, many patients with uncontrolled asthma improved to a well controlled or totally controlled level after stepwise increase of fluticasone or fluticasone combined with salmeterol. In line with GOAL, the Global Initiative for Asthma (GINA) in 2006 emphasized the importance of asthma control, and also defined asthma control on the basis of a composite measure of clinical indices. Surprisingly, a marker of inflammation was not included in the definition of asthma control, whereas chronic airway inflammation is the hallmark of asthma.

Airway inflammation does not correlate well with the level of lung function or with the symptoms of asthma(2). The question therefore arises as to whether well- or totally controlled asthma based on clinical criteria alone sufficiently reflects an adequate control of the underlying airway inflammation. One can further argue that, for an individual patient, it is not only important to have reduced airway inflammation, but also to have an optimal quality of life(3). In our opinion, a more thorough insight is needed into the complex interplay between clinical control of asthma, quality of life and the underlying airway inflammation. In this observational study we investigated airway inflammation and quality of life in subjects with totally controlled, well controlled, and uncontrolled asthma, defined according to the strict GOAL criteria.

METHODS

SUBJECTS

Smoking and non-smoking asthma patients, aged 19 to 71 years, some of whom using inhaled corticosteroids, were recruited from research cohorts investigated earlier by our group. All subjects had a doctor's diagnosis of asthma and documented bronchial hyperresponsiveness to histamine (Provocative concentration of histamine causing a 10% fall in forced expiratory volume in one second (FEV₁) from baseline (PC₁₀ histamine) ≤ 16 mg/mL, using 30-second tidal breathing). Patients were included if they had a provocative concentration of adenosine-5'-monophosphate causing a 20% fall in FEV₁ from baseline (PC₂₀AMP) ≤ 320 mg/mL. If PC₂₀AMP was higher, an additional histamine provocation challenge was performed within 2 weeks and subjects had to demonstrate a PC₁₀ histamine ≤ 16 mg/mL. The main exclusion criteria were: FEV₁ < 1.2 L, bronchiectasis, upper respiratory tract infection (e.g. colds) and/or use of antibiotics or oral corticosteroids within 2 months prior to inclusion in the study. The local medical ethics committee approved the study protocol and all subjects gave their written informed consent.

STUDY DESIGN

This prospective cross-sectional study involved the subjects paying four visits to the clinic. At visit 1, written informed consent was obtained and patients were enrolled in the study if they fulfilled the inclusion criteria. Patients were instructed how to keep a diary and how to measure peak expiratory flow (PEF) at home for the next four weeks. At visit 2 (four weeks later), clinical control of asthma was assessed according to the GOAL criteria (table 1 in supplement for Web-only publication) and the quality of life was determined using the Asthma Quality of Life Questionnaire (AQLQ). Blood was drawn for measurements of eosinophils and atopy. Then reversibility of FEV₁ was carried out with 800 μ g of inhaled albuterol (Ventolin) followed by sputum induction. Within two weeks, at visit 3, hyperresponsiveness to AMP was assessed. One to two weeks later this was followed by exhaled nitrogen monoxide (NO) measurements and a bronchoscopy (visit 4).

ATOPY

The Phadiatop screening test was used to determine atopic status; it was performed on the ImmunoCap system, according to the manufacturer's instructions (Phadia AB, Sweden). The results are presented as quotients (fluorescence of the serum of interest divided by the fluorescence of a control serum). A positive Phadiatop was defined as patient serum/control serum >1.

ASTHMA QUALITY OF LIFE

The Dutch version of the Asthma Quality of Life Questionnaire (AQLQ) was used for this study(4). The AQLQ is scored on a 7-point scale, with 32 questions on four domains: symptoms, responses to environmental stimuli and the need to avoid them, limitation of activities, and emotional dysfunction.

LUNG FUNCTION

FEV₁ was measured with a calibrated water-sealed spirometer according to standardized guidelines(5). Reversibility of FEV₁ %-predicted was measured before and after administration of 800 µg of albuterol. Provocation tests were performed using a method adapted from Cockcroft and co-workers(6). After an initial nebulized saline challenge, subjects inhaled doubling concentrations of AMP (0.04 to 320 mg/mL) by 2-min tidal breathing and at 5-min intervals. The slope of the AMP curve was calculated as the ratio between the change in FEV₁ (difference between the values at baseline and at final dose of AMP) and the final dose of AMP. Bronchial hyperresponsiveness to histamine was tested as reported previously(7), using 30-second tidal breathing and doubling concentrations ranging from 0.13 to 32 mg/mL.

SPUTUM INDUCTION AND SPUTUM PROCESSING

Sputum was induced by inhalation of hypertonic saline aerosols as previously described(8). Hypertonic saline (5%) was nebulized over three consecutive periods of 5 minutes. Whole sputum samples were processed according to a method modified from that of Fahy and colleagues(9,8). May Grünwald Giemsa (MGG) staining was used to obtain cell differentials from a total of 600 viable, non-squamous cells. The sputum was not evaluated if the percentage of squamous cells was >80% or if the total number of non-squamous cells was <600.

ALVEOLAR AND BRONCHIAL NITRIC OXIDE

Exhaled NO measurement was performed at multiple flow rates (30 mL/s, 50 mL/s, 100 mL/s and 200 mL/s) on a NIOX (Aerocrine, Stockholm, Sweden). The mean eNO value (ppb) of three technically acceptable attempts per flow rate was used for analysis. The alveolar NO fraction (ppb) as well as the bronchial NO flux (nL/s) were calculated with a modification of the two-compartment model of NO exchange by Tsoukias and George(10,11).

COLLECTION, PROCESSING, IMMUNOHISTOCHEMICAL STAINING AND QUANTIFICATION OF BRONCHIAL BIOPSIES

After local anesthesia, bronchial biopsies were obtained using a flexible bronchoscope (Olympus BF P20 or BF XT20) from segmental divisions of the main bronchi. The biopsies were fixed in 4% formalin, processed and embedded in paraffin. Bronchial biopsies were cut into 3 µm thick sections. The inflammatory profile was assessed with specific antibodies against a large panel of inflammatory markers. The inflammatory cells were quantified using computer-assisted image analysis (See Methods section in the supplement for Web-only publication for more details).

STATISTICS

All analyses were performed using SPSS (version 14.0.1; SPSS Inc, Chicago, IL, USA). The differences in the continuous variables between groups were tested with the Mann-Whitney U-test for non-normally distributed data and with the independent

sample t-test for normally distributed data. PC₂₀AMP, slope of AMP, blood, sputum and bronchial eosinophils, and eNO were transformed logarithmically to normalize their distribution. We compared the groups' dichotomous variables using the chi-squared test and performed multiple logistic regression analysis to investigate which variables were significant determinants for asthma control (see Results section in the supplement for Web-only publication). We used principal component analysis to determine the inter-relationships between different variables that might determine asthma control (see Methods section in the supplement for Web-only publication).

RESULTS

PATIENT CHARACTERISTICS

One hundred and eleven asthmatic patients with a median age of 50 (range 19-71) years were included. The reasons for not having totally controlled or well controlled asthma are given in the online supplement (Table E1). Twenty-two subjects (20%) were totally controlled, 47 (42%) well controlled, and 42 (38%) uncontrolled.

TABLE 1: BASELINE CHARACTERISTICS

	Controlled Asthma		Uncontrolled Asthma
	Totally controlled (n=22)	Well controlled (n=47)	Uncontrolled (n=42)
Sex (Male/Female)	10/12	21/26	22/20
Age (years)	48 (19-64)	52 (22-71)	48 (22-68)
Atopy (Phadiatop ratio)	4.93 (0.13-96.5)	5.56 (0.06-106)	27.3 (0.15-128)* [#]
Current smokers n (%)	7 (32)	12 (26)	10 (24)
Ex-smokers n (%)	13 (59)	27 (57)	25 (60)
Pack years in all subjects	0.9 (0-31)	2.0 (0-64)	0.2 (0-45)
Patients using ICS, n (%)	5 (23)	26 (55)	30 (71)**
¹ dose (μg/day) in all subjects	0 (0-1000) [^]	200 (0-1200)	450 (0-1000)* ^{###}
¹ dose (μg/day) in subjects using ICS	500 (400-2000)	1000 (200-2000)	800 (100-2000)
Patients using LABA, n (%)	3 (14)	15 (32)	16 (38)
FEV ₁ (% predicted)	107 (78-122) [^]	96 (42 - 132)	98 (56-135) [#]
FEV ₁ /FVC (%)	80 (58-97) [^]	74 (39-88)	73 (40-97) [#]
Reversibility FEV ₁ (%)	8.7 (0.7-23.9)	9.0 (1.5-23.3)	10.7 (-2.2 - 38.4)
PC ₂₀ AMP (mg/mL)	640 (8.1-640)	100 (0.1-640)	7.7 (0-640)*** ^{†††###}

Values are medians (ranges), unless stated otherwise. ¹dose equivalent of beclomethasone. ICS denotes inhaled corticosteroids, LABA long-acting β₂-agonist, PC₂₀AMP the provocative concentration of adenosine-5'-monophosphate causing a 20% fall in FEV₁, reversibility FEV₁ (%) change in FEV₁ expressed as increase in percentage predicted after 800 μg of Albuterol.

* p<0.05 or ** p<0.01 or *** p<0.001 versus controlled

^{†††} p<0.001 versus well controlled

[#] p<0.05 or ^{###} p<0.001 versus totally controlled

[^] p<0.05 versus well controlled.

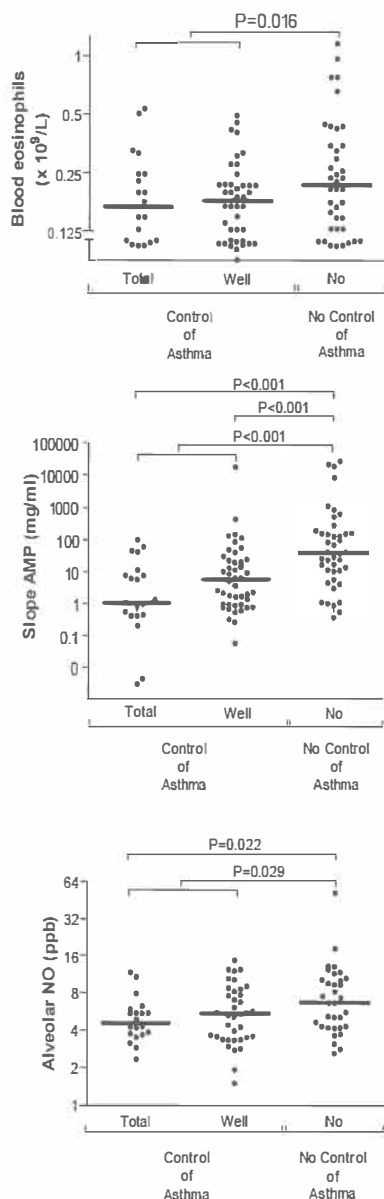


Figure 1. Biological parameters assessed in asthmatics.

Peripheral blood eosinophils (upper panel), slope of AMP (middle panel), and alveolar nitric oxide concentrations (lower panel) in totally controlled, well controlled and uncontrolled asthmatics. Each dot represents one subject. Horizontal bars represent median values. AMP denotes adenosine-5'-monophosphate, NO nitric oxide.

MARKERS OF INFLAMMATION

The 69 subjects with controlled asthma (totally and well controlled combined) had lower median blood eosinophil numbers than uncontrolled subjects: 0.18 (range 0.01–0.54) versus 0.22 (0.06–1.16) $\times 10^9/L$ ($p < 0.05$) (Fig. 1). The slope of PC20AMP was lower in asthmatics who were totally controlled or well controlled than in subjects with uncontrolled asthma: 3.8 (–0.4–17750) versus 39.7 (0.4–28000) mg/mL ($p < 0.05$). The slope of PC20AMP tended to be higher in totally controlled subjects than in well controlled subjects ($p = 0.08$) (Fig. 1). Alveolar NO levels were lower in subjects with controlled asthma than in uncontrolled subjects: 5.3 (1.5–14.9) versus 6.7 (2.6–51.7) ppb, $p < 0.05$ (Fig. 1).

In sputum, both the total number and proportion (%) of inflammatory cell types did not differ significantly between controlled and uncontrolled subjects (Table E2 in the online supplement). Post-hoc analysis in subgroups of asthmatics divided according to current smoking status and current corticosteroid use did not affect these results.

Subjects with controlled asthma had a lower level of eosinophil activation, as determined by EPX (eosinophil peroxidase)-immunopositivity in bronchial biopsies, than uncontrolled subjects (Table 2, Fig. 2). Controlled asthma was accompanied by an increase in intact epithelium compared to uncontrolled asthma (Table 2, Fig. 2). All the other inflammatory and remodeling variables we investigated were comparable in controlled and uncontrolled subjects (Tables 2 and E3 in the online supplement).

MULTIPLE LOGISTIC REGRESSION ANALYSIS ON ASTHMA CONTROL

Multivariate logistic regression analysis was used to reveal which variables determine the control of asthma. Due to multi-co-linearity all the variables related to eosinophilic inflammation could not be entered simultaneously into the model. We therefore created a model including sex, age, Phadiatop ratio, inhaled corticosteroid (ICS) use and FEV₁/VC, with the other variables (alveolar NO, blood eosinophils, AMP slope, EPX-positive pixels or the percentage intact epithelium) being entered separately.

TABLE 2: INFLAMMATION AND REMODELING IN BIOPSIES

	Controlled asthma		Uncontrolled asthma
	Totally controlled n=19	Well controlled N=33	Uncontrolled n=37
Inflammatory cells			
Eosinophils	2 (0-26)	1 (0-21)	2 (0-32)
Mast cells	12 (3-24)	8 (0-26)	9 (0-17)
Macrophages	15 (0-30)	12 (0-47)	11 (0-57)
Neutrophils	9 (0-46)	5 (0-34)	6 (0-20)
T lymphocytes (CD3 ⁺)	68 (23-294)	55 (13-177)	66 (4-219)
T lymphocytes (CD4 ⁺)	24 (4-70)	16 (0-67)	17 (0-71)
T lymphocytes (CD8 ⁺)	25 (4-139)	17 (0-136)	23 (1-205)
Inflammatory cell activation			
EPX immunopositivity	157 (16-1268)	101 (6-1787)	219 (19-5312)* ^{††}
Mast cell degranulation (%)	80 (22-100)	69 (25-100)	75 (0-100)
Remodeling			
BM thickness (μm)	6.3 (4.3-9)	5.2 (3.6-8.7)	5.9 (2.8-12.6)
Intact epithelium (%)	13 (0-32.8)	10.5 (0-65.3)	5.6 (0-47.6)*

Inflammatory cells are expressed as the number per 0.1 mm². Values are medians (ranges). * p<0.05 versus controlled, ^{††} p<0.01 versus well controlled.

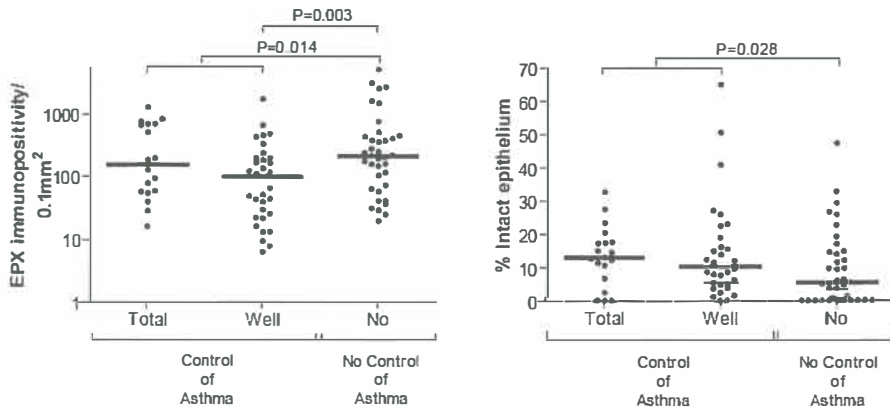


Figure 2. Biopsy parameters assessed in asthmatics.

EPX (eosinophil peroxidase) immunopositivity (left panel) and the percentage of intact epithelium (right panel) in bronchial biopsies totally controlled, well controlled and uncontrolled asthmatics. Each dot represents one subject. Horizontal bars represent median values.

This showed that a higher Phadiatop ratio and ICS use were significant determinants of worse asthma control in most of the models. It was noteworthy that a higher AMP slope (OR: 0.39; 95%CI: 0.24-0.63) and higher EPX-positive pixel numbers (OR: 0.32; 95%CI: 0.13-0.79) were significant determinants for worse asthma control (see Results section in the supplement for Web-only publication).

COMPARISON WITH ASTHMA QUALITY OF LIFE (AQLQ)

Subjects with controlled asthma demonstrated higher median scores in the total AQLQ than uncontrolled subjects: 6.7 (range 5.0-7.0) versus 5.9 (3.7-7.0) ($p < 0.001$) (Fig. 3). They also had significantly higher scores in all domains of the AQLQ: symptoms 6.7 (4.9-7.0) versus 5.5 (3.7-7.0), activity 6.8 (4.5-7.0) versus 6.3 (3.9-7.0), emotion 7.0 (5.0-7.0) versus 6.6 (3.8-7.0) and environment 6.5 (3.8-7.0) versus 5.5 (3.0-7.0). AQLQ scores did not differ significantly between the totally and well controlled subjects.

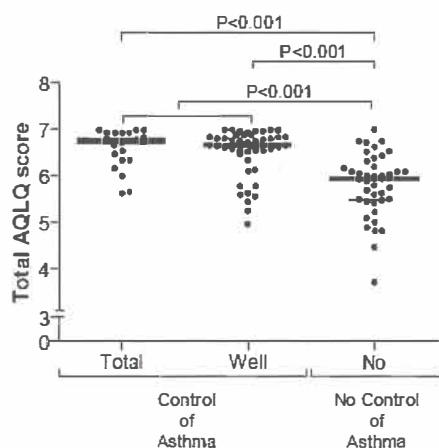


Figure 3. Asthma quality of life as assessed with the Asthma Quality of Life Questionnaire.

The AQLQ (Juniper et al⁴) was given to totally controlled, well controlled and uncontrolled asthmatics. It is divided into four domains: symptoms, activities, emotions, environmental. Each dot represents one subject. Horizontal bars represent median values.

DISCUSSION

The Global Initiative for Asthma (GINA, 2006) defined control of asthma by a composite measure of clinical findings but without including markers of airway inflammation, the hallmark of asthma(12). Our study is the first to have investigated whether the level of asthma control according to GINA is associated with both direct and indirect markers of airway inflammation including airway wall biopsies in a large group of asthmatics with a wide spectrum of disease severity. Patients with totally controlled and well controlled asthma demonstrated less hyperresponsiveness to AMP, lower NO levels in exhaled air, lower eosinophil numbers in peripheral blood, lower activated eosinophil numbers in the bronchial wall, a higher degree of epithelial intactness in the bronchial wall, and higher quality of life scores than uncontrolled subjects.

Our data show that control of asthma is associated with a lower degree of airway inflammation as assessed by indirect markers like PC20AMP, exhaled NO, and peripheral blood eosinophils. Even more importantly, we found that control of asthma was associated with a lower degree of airway inflammation as assessed by the pathological gold standard of asthma, namely the mucosal infiltration of activated eosinophils and loss of epithelial integrity. Thus, the concept of a composite measure for clinical asthma control, combining different variables like night- and daytime symptoms, PEF, and use of rescue medication, appears to be valid, or at least to have an underlying pathobiological substrate.

We have further demonstrated that different aspects of inflammation (nitric oxide, eosinophils, epithelial cells, PC20AMP) stemming from different parts of the lung (exhaled air, blood, bronchial biopsies) are associated with control of asthma. This is not particularly surprising since all these inflammatory factors interact and contribute to the overall, general inflammation that is present in asthma. On the other hand, the heterogeneity of asthma with different inflammatory subtypes is increasingly acknowledged(13,14). Our study also lends support to this concept, as we found that less severe hyperresponsiveness to AMP, lower eosinophil numbers, and better epithelial integrity are all independent factors contributing to higher asthma control. Regardless of the exact relationship, our results suggest that aiming for clinical control of asthma does suppress the underlying inflammation. Randomized intervention studies with a longitudinal study design are needed to further demonstrate a more causal and specific relationship.

We had three unexpected findings in this study. First, smoking history did not contribute significantly to the level of asthma control, despite smoking being associated with poorer asthma control in two other studies(15,16). This could be explained by a "healthy smoker" effect, i.e. those with relatively healthy airways are able to tolerate smoke inhalation, to persist in their smoking habit, and to show good control of their asthma.

Secondly, ICS use contributed to the variation in asthma control, but in an opposite direction to that expected, with a significantly higher percentage of ICS users and dose of ICS used in the uncontrolled group. Indeed, an "unhealthy ICS" effect may well have been present in our study, since it is well-known that some asthmatics do not respond well to corticosteroids(17,18). This was also found in the GOAL study, which showed that 20-30% of the participants did not improve their baseline level of uncontrolled asthma despite long-term administration of increasing doses of ICS.

Thirdly, control of asthma did not significantly associate with lower sputum eosinophils, opposite to two other studies(19,20). Apart from a different asthma population the two studies also used different tools to assess asthma control. Quaedvlieg et al(19) used the shortened Asthma Control Questionnaire that does not take lung function into account(21), whereas Romagnoli et al(20) included variation of PEF in stead of PEF itself(22). Both studies measured asthma control during one week only, and not as a strict composite measure during 4 weeks as we did. Our study shows that other inflammatory measures relate to the strictly used definition of asthma control, and particularly eosinophilic makers in peripheral blood and airway wall biopsies. It is an intriguing finding that control of asthma significantly associates with lower peripheral

blood eosinophils and lower activated eosinophil numbers in the bronchial wall, yet not with sputum eosinophils. We do not have an explanation for this discrepancy, but if true, it is questionable whether sputum eosinophils can be used to monitor control of asthma.

The health-related quality of life in our patients with controlled asthma was significantly better than in those with uncontrolled asthma. As the difference between the two groups was higher than the minimal, clinically important difference of 0.5 point, it is also more attractive from the patients' perspective to aim for a better level of control in those without adequate control(23). In addition, we observed a ceiling in the quality of life measures between the totally controlled and well controlled subjects, suggesting that aiming for the best level of asthma control does not further improve an individual's quality of life.

The participants in this study are not fully comparable to those in the larger GOAL study(24), which included only subjects with uncontrolled asthma, whereas we also included subjects with totally controlled and well controlled asthma. The GOAL study also included younger subjects (including children) and had a lower prevalence of ex-smokers, lower lung function, and higher bronchodilator reversibility. When we compared the reasons why patients failed to achieve asthma control in the GOAL study and in our study, there were a few striking differences. The percentages of patients failing to achieve control due to the GOAL criteria were 63% due to awakening in GOAL vs our study 50%, daytime symptoms (95% vs 29%), rescue medication (92% vs 48%), and PEF >80% predicted (72% vs 76%). Thus, the prevalence of daytime symptoms and rescue medication in the GOAL study were considerably higher than in our study. It is possible that the patients in the GOAL study had more severe disease, having a lower baseline lung function and more symptoms than our patients. Larger, explorative studies will be needed to determine why some patients do not achieve total control of their asthma and this may well have important consequences for the therapies prescribed.

In conclusion, our observational study demonstrates that clinical control of asthma is associated with both direct and indirect markers of airway inflammation. Moreover, better asthma control associates with a higher quality of life for the patients. Thus, aiming for good clinical control of asthma, as recommended by the GINA guidelines, is important from the patients' point of view and suppresses the underlying airway inflammation as well.

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Clinical control of asthma is associated with measures of airway inflammation

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Online data supplement

ONLINE SUPPLEMENT: METHODS

COLLECTION, PROCESSING AND IMMUNOHISTOCHEMICAL STAINING OF BRONCHIAL BIOPSIES

The inflammatory profile of 11 asthmatic subjects was assessed with specific antibodies against eosinophilic peroxidase (EPX, laboratories of NA Lee and JJ Lee, Mayo Clinic, Scottsdale, Arizona, USA), mast cell tryptase (AA1, DAKO, Glostrup, Denmark), macrophages (CD68, DAKO), neutrophil elastase (NP57, DAKO) and T-lymphocytes (CD3 (DAKO), CD4 (Novocastra, Newcastle upon Tyne, UK) and CD8 (DAKO)). In short, sections were deparaffinized, antigens were retrieved, and the sections then incubated with the primary antibodies. These antibodies were detected with Envision™ Detection Kit (DAKO) followed by the chromogen NovaRED (Vector Labs, Burlingame, California, USA). EPX and CD8 were detected using biotinylated anti-mouse IgG1 (Southern Biotechnology, Birmingham, Alabama, USA) and alkaline phosphatase- (DAKO) or peroxidase-labeled streptavidine conjugates (DAKO), followed by permanent Red (DAKO) and NovaRed chromogens, respectively. Epithelial proliferation was assessed by Ki67 expression and submucosal deposition of extracellular matrix (ECM) by collagen 3 expression. In short, sections were deparaffinized, antigens were retrieved, and the sections incubated with antibodies against Ki67 (DAKO) and collagen 3 (Southern Biotechnology). These antibodies were detected with two peroxidase-labeled conjugates (both DAKO). 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used since chromogen and hematoxylin (Ki67) or methylgreen (collagen 3) were used for counterstaining. All stainings (except for Ki67) were performed in an automated system using the DAKO autostainer in three consecutive runs per cell marker. Sections were manually counterstained with methylgreen.

QUANTIFICATION

Quantification of inflammatory cells was performed by a blinded observer using computer-assisted image analysis at a magnification of 200x (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). The largest of three biopsy samples was generally chosen for quantification. The number of positively stained inflammatory cells was counted in the submucosal area 100 µm under the basement membrane (BM), in a total area of 0.1 mm² per biopsy sample. Additionally, activation of eosinophils and mast cells was determined by their degree of degranulation. EPX staining showed widely spread distribution of eosinophilic granules, not necessarily in close proximity to EPX+ cells. Therefore, degranulation of eosinophils was determined by quantification of the EPX immunopositive area by computer-assisted image analysis. AA1+ mast cell granules were only observed in close proximity to AA1+ cells and, therefore, the percentage of degranulated AA1+ cells was determined by counting (Carroll, N.G. et al. *Eur. Respir. J.*, 2002. 19:879-885). In short, positively stained nucleated mast cells were classified as intact if they were dense, compact, had unbroken boundaries, and did not have any surrounding positively stained granules. All other nucleated AA1+ cells were classified as degranulated.

BM thickness was calculated by dividing the BM surface area by the BM length. Epithelial integrity was determined using hematoxylin and eosin-stained biopsy sections and expressed as a percentage of the BM covered with: (1) normal epithelium (a layer of basal and ciliated columnar epithelial cells without detachment from the BM), (2) basal epithelial cells only, (3) no epithelium: denuded BM, or (4) metaplastic epithelium (multilayered epithelium covered by a flattened layer of squamous epithelial cells and absence of ciliated cells).

The number of goblet cells was counted on PAS-stained biopsy sections and expressed per mm of BM. Additionally, the number of PAS-positive pixels was determined in the epithelium and expressed as the percentage of mucus-positive epithelium per biopsy section. Epithelial thickness was determined by dividing the epithelial surface area by the BM length. Epithelial proliferation was determined by counting the number of

Ki67-positive epithelial nuclei in intact and basal epithelium. Expression of collagen 3 was measured by computer-assisted image analysis in the submucosal area 200 μm under the BM, and was expressed as the percentage of positive tissue per biopsy section.

STATISTICS

Principal component analysis was used to determine the inter-relationships between different variables that might determine asthma control, i.e. questionnaires, lung function inflammation, and remodeling. Exploratory principal component analysis included the following variables: postbronchodilator FEV₁/VC (%), postbronchodilator reversibility (% predicted), morning PEF (% predicted), Asthma Quality of Life Questionnaire (AQLQ) total score, AQLQ symptoms score, self-reported rescue medication usage, daytime symptoms, and night-time awakenings, slope AMP, alveolar NO (ppb), smoking history in pack-years, inhaled corticosteroid (ICS) use, atopy (Phadiatop ratio), number of eosinophils in blood, sputum (number) and bronchial biopsy (EPX immunopositivity), bronchial mast cells (number), and intact epithelium (%). Two tailed p-values < 0.05 were considered statistically significant.

ONLINE SUPPLEMENT: RESULTS

MULTIPLE LOGISTIC REGRESSION ON ASTHMA CONTROL

Multivariate logistic regression analysis was used to reveal which variables determine the level of control of asthma. However, due to multi-co-linearity, not all the variables related to eosinophilic inflammation (alveolar NO, slope AMP, eosinophils in sputum and EPX pixels in biopsies) could be entered simultaneously into the model. We therefore created an alternative model including sex, age, Phadiatop ratio, inhaled corticosteroid (ICS) use and FEV₁/VC. We entered the following variables separately into this model: alveolar NO, blood eosinophils, slope AMP, EPX-positive pixels or the percentage intact epithelium. In most models (4 out of 5) Phadiatop ratio and ICS use were found to be significant determinants of asthma control. The two exceptions were when alveolar NO was entered, ICS use was no longer a significant determinant, and when slope AMP was entered, Phadiatop ratio was no longer a significant determinant for asthma control. Importantly, the AMP slope (odds ratio (OR) 0.39; 95% confidence interval (CI) 0.24-0.63) and EPX-positive pixels (OR 0.32; 95%CI: 0.13-0.79) were also found to be significant determinants for asthma control.

PRINCIPAL COMPONENT ANALYSIS

Due to the multi-co-linearity, we decided to explore our dataset using principal component analysis for inter-relationships between the different variables that might determine asthma control, i.e. questionnaires, lung function, and airway inflammation. Principal component analyses yielded six separate factors that accounted for 66.9% of the total variance. Factor 1 comprised the AQLQ total score, AQLQ symptoms score, self-reported daytime symptoms, and night-time awakenings. Factor 2 included eosinophils in blood (number), sputum (number) and bronchial biopsy (EPX immunopositivity), alveolar NO (ppb), and self-reported rescue medication usage. Factor 3 contained postbronchodilator FEV₁/VC (%) and morning PEF (% predicted). Factor 4 included atopy (Phadiatop ratio), slope AMP and postbronchodilator reversibility (% predicted). Factor 5 contained intact epithelium (%), bronchial mast cells (number), and ICS use. Factor 6 comprised only smoking history (pack-years).

TABLE E1. FAILURE OF ASTHMA CONTROL DURING 4 WEEKS FOLLOW-UP

	Criteria of asthma control		No of patients that failed the criteria ^ψ			Median (range) values per group		
	Totally Controlled	Well Controlled	Total (n=22)	Well (n=47)	No (n=42)	Total	Well	No
During 4 weeks	All of:	Two or more of:						
Daytime symptoms n (%) [¶]	None	≤ 2 days with score >1	0	26 (55)	12 (29)	0 ^{^^}	0.18 (0-1.4)	1.0 (0-4.0) ^{*** †††###}
Rescue beta-2 agonist use n (%) [¶]	None	≤ 2 days & ≤ 4 occasions	0	9 (19)	20 (48)	0 ^{^^}	0 (0-2)	0.4 (0-3.3) ^{*** †††###}
Morning PEF (%) [¶]	≥ 80% pred. every day	≥ 80% pred. every day	0	24 (51)	32 (76)	97 (88-124) ^{^^}	87 (41-128)	81 (40-99) ^{***###}
AND each week all of the following								
Night-time awakening [†]	None	None	0	0	21 (50)	0	0	2 (0-41) ^{*** †††###}
Exacerbations [†]	None	None	0	0	0	0	0	0
Emergency visits [†]	None	None	0	0	0	0	0	0
Treatment-related AEs [†]	None enforcing change in asthma therapy	None enforcing change in asthma therapy	0	0	0	0	0	0

^ψNumbers of occasions that patients failed to fulfil the criteria of totally controlled or well controlled. Values in the well controlled asthma group are the number of occasions that patients do not fulfil the criteria of total control. Values in the uncontrolled asthma group are the number of occasions that patients do not fulfil the criteria of being well controlled. [¶] average per day; [†] number of events during 4 weeks. Daytime symptom score: symptoms for 1 short period during the day (overall scale: 0 [none] to 5 [severe]). Exacerbations: deterioration in asthma requiring treatment with oral corticosteroid or visit to emergency department, hospitalization, or both. AE adverse event; ^{***} p<0.01 versus controlled; ^{†††} p<0.001 versus well-control; ^{##} p<0.01 or ^{###} p<0.001 versus totally control; ^{^^} p<0.01 or ^{^^^} p<0.001 versus well controlled.

TABLE E2: INFLAMMATION IN SPUTUM

	Controlled Asthma		Uncontrolled Asthma
	Totally controlled (n=22)	Well controlled (n=47)	Uncontrolled (n=42)
<i>Sputum evaluable</i>	13 (59%)	34 (72%)	33 (79%)
Total cells ($\times 10^6/\text{mL}$)	0.47 (0.07 – 1.79)	0.54 (0.05 – 3.00)	0.37 (0.14 – 3.86)
Neutrophils (%)	60.9 (21.6 – 84.9)	59.7 (15.6 – 94.0)	55.8 (16.8 – 88.8)
Macrophages (%)	34.1 (13.1 – 76.5)	36.3 (4.9 – 82.5)	35.1 (9.1 – 66.7)
Eosinophils (%)	1.4 (0.0 – 5.7)	0.8 (0.0 – 38.6)	1.2 (0.0 – 67.1)
Lymphocytes (%)	0.2 (0.0 – 2.4)	0.5 (0.0 – 2.4)	0.3 (0.0 – 5.7)

Values are medians (ranges). No significant differences between controlled versus uncontrolled groups, or between totally controlled versus well controlled. Absolute numbers of inflammatory cells in sputum were also similar between the three groups.

TABLE E3: REMODELING IN BRONCHIAL BIOPSIES

		Controlled Asthma		Uncontrolled Asthma
		Totally controlled (n=19)	Well controlled (n=33)	Uncontrolled (n=37)
Epithelium	Goblet cells (N)	37 (4-85)	35 (2-92)	39 (9-119)
	Mucus (%)	8 (0.8-21.2)	13 (0.9-33.9)	9.4 (0.2-46.1)
	Intact epithelium (%)	13 (0-32.8)	10.5 (0-65.3)	5.6 (0-47.6)*
	Basal epithelium (%)	37.2 (14.7-74.3)	40.9 (5.1-80.2)	42.5 (18.5-72.2)
	Denuded BM (%)	16 (0-58.8)	9.6 (0.7-47.6)	17.5 (2.1-63.2)
	Metaplasia (%)	0 (0-26.2)	0 (0-18.2)	0 (0-19.4)
	Thickness (μm)	17.3 (11.2-36.8)	20.4 (7.5-91.4)	17.6 (7.9-48.1)
	Ki67 ⁺ (% intact)	5.1 (0.4-11.2)	4.4 (0.9-20.1)	4.0 (0-15.5)
Submucosa	Ki67 ⁺ (% basal)	2.7 (0-8.5)	5.1 (0.4-28.9)	3.8 (0-25.5)
	Collagen 3 (%)	45.4 (8.2-64.5)	39 (8.6-77.9)	42 (15.5-64.9)

Values are medians (ranges). N=number

* $p < 0.05$ versus control.

Chapter 8

Summary and Discussion

Chapter 8

The aim of this thesis was to gain more insight in processes underlying progression and remission of asthma. The research was focussed on inflammation and remodeling, effects of smoking, and surrogate markers of asthma control.

REMISSION OF ASTHMA

To investigate if remission of asthma really exists we tested markers of airway inflammation and remodeling in steroid naive, non-smoking subjects meeting the criteria for complete asthma remission. Complete asthma remission was defined by absence of asthma symptoms and medication, combined with a normal lung function and absence of bronchial hyperresponsiveness.

We compared inflammatory cells in induced sputum in current asthmatics and former asthmatics who had complete remission (Chapter 2). No differences in sputum inflammatory cell percentages were found between both groups. This lack of difference may have been caused by the mild disease in the current asthma group that was responsive to histamine but nevertheless did not use inhaled steroids. Interestingly, we found differences between subjects with current asthma and complete asthma remission when percentages of sputum inflammatory cells after AMP provocation were compared. A significant increase in the percentage of sputum eosinophils was observed in current asthmatics, even in those without hyperresponsiveness to AMP, i.e. in the situation that they inhaled comparably high concentrations of AMP as those who had asthma in complete remission and hence no response to AMP. In contrast, no increase in sputum eosinophils was found in subjects meeting criteria for complete asthma remission.

Although group sizes were relatively small, the lack of induction of sputum eosinophilia by the AMP provocation in this group was promising as to our concept of complete asthma remission. It was therefore a logical and important step for the study to assess if subjects meeting the criteria for complete asthma remission really had outgrown their asthma, as shown by normal airway wall tissue.

In the third chapter, the concept of complete asthma remission was further investigated with the addition of bronchial biopsy data. A larger group of individuals meeting the criteria for complete asthma remission was included. Subjects with complete asthma remission were compared with subjects in clinical remission, i.e. asthmatics without asthma symptoms or medication, and with subjects having current asthma. This time, smokers were included as well. In the group with current asthma, both subjects with and without inhaled corticosteroid use were investigated. The first main finding was that eosinophil numbers were significantly lower in bronchial biopsy tissue and in peripheral blood in the group with complete asthma remission. Just as observed in the second chapter, no differences in sputum eosinophil percentages were found. The second important finding was that basement membrane thickness was comparable in complete and clinical asthma remission. In contrast, subjects with current asthma using inhaled corticosteroids had reduced basement membrane thickness. This finding is in line with previous studies showing that inhaled corticosteroids reduce basement membrane thickness(1;2).

Thus, it seems that complete remission of asthma does exist in the context of remaining remodeling features, given the observed persistence of basement membrane thickening. It is intriguing that, in our study, loss of bronchial hyperresponsiveness exists despite the presence of this remodeling marker. It is thus likely that active eosinophilic inflammation with associated epithelial damage, edema and a primed state of the inflammatory response system contributes more to bronchial hyperresponsiveness than basement membrane thickness itself. This is supported by the higher eosinophil peroxidase (EPX) immunopositivity in the airway wall of current asthmatics, which we found. Additionally, differences in the volume of airway smooth muscle or the absence of activated mast cells between smooth muscle bundles could have contributed to loss of bronchial hyperresponsiveness in complete remission of asthma. These differences were not further investigated in our studies.

AMP RESPONSIVENESS

In chapter two and chapter four we investigated the characteristics of current asthma patients with respect to presence or absence of AMP responsiveness. In chapter two we noticed that current asthma patients who were hyperresponsive to histamine but not to AMP showed less increase in sputum eosinophil percentages after an AMP challenge compared to asthma patients who were responsive to both provocation tests. This increase in eosinophil percentage was significantly higher in AMP responsive asthmatics despite inhaling a lower cumulative dose of AMP as compared to AMP unresponsive asthmatics. The prevalence of atopy was significantly higher in AMP responsive subjects, as well as the level of atopy as assessed by the Phadiatop test.

In chapter four we showed that eosinophils were significantly more present in bronchial biopsies from the AMP responsive group. Additionally, the basement membrane was thicker in AMP responsive asthmatics. More severe AMP responsiveness was associated with higher percentages of eosinophils in the airway wall, whereas the number of mast cells in bronchial submucosa was similar between both groups. Likewise, the severity of AMP responsiveness and basement membrane thickness were significantly correlated. However, a direct causal relation between the latter two is uncertain, since both could well be markers of increased (eosinophilic) airway inflammation.

To our knowledge, the study described in chapter is the first investigating bronchial biopsies in relation to AMP responsiveness. In our small group of asthma patients we show that a positive AMP responsiveness is not only related to markers of airway inflammation like elevated eosinophils in sputum and higher EPX immunopositivity in bronchial mucosa, but also to markers of airway remodeling like metaplastic epithelium and increased basement membrane thickness. This could imply that positive AMP responsiveness in asthma patients may be an extra factor in the decision for treatment with inhaled corticosteroids. First, because positive AMP hyperresponsiveness is associated with increased eosinophilic inflammation and active remodeling, and second because this same eosinophilic type of inflammation is usually associated with a good response to corticosteroids.

Another important observation in these chapters was made in asthma patients who are unresponsive to AMP, despite a positive response to histamine. These AMP unresponsive asthmatics had lower eosinophilic inflammation, less thickening of basement membrane and less metaplastic epithelium compared to AMP responsive asthmatics. This observation could imply that unresponsiveness to AMP is a marker of mild inflammatory asthmatic disease. However, not all AMP unresponsive individuals show this low eosinophilic inflammation and remodeling. Thus, further research is needed to determine if absence of AMP responsiveness also has clinical consequences for individual asthma patients.

DECLINE IN LUNG FUNCTION

In chapter five, we compared asthma patients meeting the clinical criteria for complete asthma remission with current asthma patients having either slow or fast decline in lung function. Slow decline was arbitrary defined as < 30 ml/year decline in FEV₁ and fast decline as > 30 ml/year. In patients with complete asthma remission the mean decline in lung function was very small, only 8 ml/year. Current asthma patients with a slow decline in FEV₁ had a mean decline of 18.6 ml/year and those with a fast decline 50.3 ml/year. Current asthma patients with a fast decline in FEV₁, had more severe hyperresponsiveness to AMP, more eosinophilia in blood and sputum, and higher sputum ECP levels compared to slow decliners and the complete remission group. However, in bronchial biopsies, no differences were found in inflammatory cells and markers for remodeling.

Sputum eosinophilia and ECP were significantly correlated with an accelerated decline in FEV₁. A positive association between blood eosinophils and FEV₁ decline in asthma

Chapter 8

has been described earlier(3). In contrast, the present study showed no association between an accelerated decline and the number of airway wall eosinophils and basement membrane thickness. Both latter findings are in line with observations by Van Rensen et al. In a 7.5-year follow-up study in 32 asthma patients, no association was found between annual decline in post-bronchodilator FEV₁ and thickness of the reticular layer or eosinophil counts in bronchial biopsies(4). Thickening of the basement membrane is already observed in childhood asthma and to a similar extent as seen in adults with asthma(5). Thus, basement membrane thickening may be an early feature of asthmatic airway remodeling, yet it seems to be unrelated to decline in FEV₁ in asthma.

A possible explanation for the lack of association between inflammatory cells in bronchial mucosa and an accelerated decline in lung function is the following. An accelerated decline in FEV₁ may be associated with increased recruitment of eosinophils from the bone marrow to the blood. Possibly, these eosinophils move straight from the blood to the luminal space of the lung and they are possibly not retained in the airway wall e.g. due to epithelial damage. In other words: bronchial biopsies give only a snap-shot of the cellular composition of the airway wall which is not identical to the traffic of inflammatory cells through the airway wall.

Together, our findings support the importance of measuring sputum eosinophils in asthma in being a predictor for accelerated decline in FEV₁. In line with findings described above, treatment with inhaled corticosteroids should be seriously considered in these patients.

ASTHMA AND SMOKING

In chapter six we investigated effects of smoking in a large group of asthma patients, including smokers, ex-smokers, and never-smokers. Research was focused on symptoms, inflammation and markers of remodeling. We found that cigarette smoking was associated with airway remodeling and that this airway remodeling correlated with the severity of self-reported asthma symptoms. Eosinophil numbers were decreased and mast cell numbers increased in current smokers compared to never smokers. Interestingly, ex-smokers did not have increased airway remodeling and mast cell numbers were significantly lower than in current smokers. Surprisingly, airway remodeling and mast cell numbers were similar to never-smokers, which suggests that smoke induced damage can be reversed when patients quit smoking.

The main difference between ex-smokers and never-smokers was the increased number of eosinophils in never-smokers compared to both other groups. In ex-smokers, the number of eosinophils was positively correlated with the duration of smoking cessation. This suggests that the down-regulatory effect of smoking on the number of eosinophils disappears after smoking cessation.

ASTHMA CONTROL, INFLAMMATION AND QUALITY OF LIFE

Markers of asthma control, inflammation and quality of life were integrated in chapter seven. A total of 69 asthma patients with controlled asthma (total and well control according to GOAL criteria) were compared to 42 asthma patients with uncontrolled asthma. We showed that patients with controlled asthma had lower median blood eosinophil numbers, slope of hyperresponsiveness to AMP, and alveolar nitric oxide levels than the subjects with uncontrolled asthma. Biopsies from subjects with controlled asthma had fewer eosinophilic granules, and more intact epithelium than uncontrolled subjects. These findings are very important, because the use of the clinical definition of asthma control by GOAL criteria is now supported by lower inflammation and remodeling in bronchial biopsies.

Health-related quality of life in our patients with controlled asthma was significantly better than in those with uncontrolled asthma. As the difference between the two groups was higher than the minimal, clinically important, difference of 0.5 point, it is also more attractive from a patients' perspective to aim for a better level of control.

FUTURE PERSPECTIVES

An important observation in our studies that needs to be investigated further is the persistent basement membrane thickening in patients meeting the clinical criteria for complete asthma remission. Is this thickening just a marker of damage that has occurred in the past or also an indicator of future relapse of asthma? Longitudinal follow-up studies are required in this group of asthma patients who are in objective clinical remission. If relapse does not occur and decline in lung function is comparable to that found in healthy controls, it may be possible to refrain from treating them with corticosteroids because the treatment has no clinical consequences. However, if relapse does occur and/or the decline in lung function is increased in (a subgroup of) these patients, further clinical follow-up and treatment is needed.

Another interesting finding is the observation that some steroid-naïve asthma patients are unresponsive to AMP, despite being hyperresponsive to histamine. These AMP unresponsive asthmatics had lower eosinophilic inflammation in sputum and bronchial mucosa, less thickening of basement membrane and less metaplastic epithelium compared to AMP responsive asthmatics. This observation could imply that there is less need for treatment with corticosteroids in AMP unresponsive asthmatics. Clearly further studies are needed to prove this concept in larger cohorts and also to gather longitudinal data in this specific group of asthma patients. Also, longitudinal studies will have to elucidate whether AMP unresponsiveness in asthma indicates an intermediate state between asthma and asthma remission and which factors drive to remission or persistence.

In our studies, the presence of a positive hyperresponsiveness test to AMP in steroid-naïve asthma patients was associated with eosinophilic inflammation, thickened basement membrane and faster decline of lung function. These findings implicate that steroid-naïve asthma patients with hyperresponsiveness to AMP are at risk for pulmonary damage. Since eosinophilic inflammation usually responds well to treatment with inhaled corticosteroids, this treatment should be given to these patients. It would be interesting to investigate if the dose of inhaled corticosteroids can be titrated according to bronchial hyperresponsiveness to AMP. If so, AMP guided therapy should be compared to other markers of airway inflammation used for treatment guidance, as hyperresponsiveness to methacholine(1), inflammatory cells in induced sputum(6), and levels of exhaled NO(7).

Another interesting question is if treatment with inhaled corticosteroids affects the decline in lung function in this group of patients. Lange et al. found that treatment with ICS was associated with a decreased decline in FEV₁ in both women and men, as well as in non-smokers and smokers in a 10-year follow up study(8). In contrast, in a 23-year follow-up study, Dijkstra et al. found that treatment with ICS was only associated with a reduction in the decline in FEV₁ in men who had smoked less than 5 pack years. No effect on decline in FEV₁ was found in women or in men with >5 pack years of smoking(9). We hypothesize that treatment with inhaled corticosteroids reduces the decline in FEV₁ in AMP responsive and steroid naïve asthma patients, because in our study AMP responsiveness was associated with eosinophilic inflammation and an accelerated decline in FEV₁. Prospective, longitudinal studies could investigate if this hypothesis is valid.

Our study on smoking and asthma contributed to better understanding the link between smoking induced changes in the airway wall and symptoms of asthma. We found that cigarette smoking increased airway remodeling and that this airway remodeling correlated with self-reported asthma symptoms. Also, an important finding was that these smoking induced changes may well be reversible after cessation of smoking. Thus, longitudinal studies are needed to investigate the effects of smoking cessation on decline of lung function in this group of asthma patients. Does the decline in lung function decrease to levels observed in never-smoking asthma patients, or to levels found in healthy controls?

It is also interesting to test if ex-smokers restore responsiveness to treatment with

inhaled corticosteroids, as steroid resistance is a known problem in asthma patients who smoke(10). Chaudhuri et al. also found, in a small group of 10 ex-smokers with asthma, that peak expiratory flow levels increased after treatment with oral corticosteroids, which supports reversibility of steroid resistance(10). More research should increase our understanding of steroid unresponsiveness in asthmatic smokers and perhaps reversibility of responsiveness after smoking cessation. It would be interesting to compare factors associated with steroid unresponsiveness as increased glucocorticoid receptor β expression, increased histone deacetylase-2 activity and increased expression of nuclear factor KB(11) between (our) groups of never-smokers, ex-smokers and current smokers.

In non-smoking asthmatics, eosinophilic inflammation is associated with steroid responsiveness. Treatment with steroids in these patients reduces the numbers of eosinophils and decreases asthma symptoms(6). In contrast, smoking in asthma also decreases eosinophil numbers, but is associated with increased inflammation, remodeling and an increase in asthma symptoms(11). Thus, suppression of the number of eosinophils can not explain the different effects on the clinical expression of asthma. Possibly, bacterial lipopolysaccharide (LPS) in cigarette smoke increases airway inflammation, because LPS is a known activator of nuclear factor KB(12). Probably, LPS and other compounds in inhaled cigarette smoke change the lower airways epithelium into a different pathological phenotype. Indeed, in our study smokers had more goblet cells, mucous positive epithelium, more epithelial proliferation, and a thicker epithelial layer compared to never-smokers. These changes in the airway epithelium may also increase airway mucosal permeability. Together with increased mucous production this may impair the deposition of inhaled antigens as well as corticosteroids within the airways. Thus, further investigation of smoke induced changes of the airway epithelium in asthma may well add to our understanding of steroid unresponsiveness in this group of patients.

In asthma, it is suggested that a structurally and functionally defective lower airway epithelium underlies abnormal responses to the inhaled environment leading to enhanced signalling between the airway epithelium and underlying structural and immune cells. Activation of this epithelial-mesenchymal trophic unit might also be responsible for tissue remodeling(13). Further research on this epithelial-mesenchymal trophic unit could increase our understanding of the different courses of asthma, e.g. stable disease, progression or remission, relapse, and responses to environmental factors as cigarette smoke.

It would be interesting to know if epithelial integrity restores in asthma remission. Is there still increased epithelial shedding? Are the epithelial tight junctions intact? Additionally, can we find markers for chronic repair in asthma remission? What is the expression of CD44 or the epithelial growth factor receptor (EGFR)? Are markers for cell proliferation as Ki-67 and proliferation cell nuclear antigen (PCNA) reduced? Comparison between asthma remission and healthy controls should be made. If longitudinal studies show that relapse occurs in a subgroup of patients with complete asthma remission, further research may reveal which factors can identify patients who really are in remission or not. Maybe, the absence or presence of markers for chronic epithelial inflammation and repair can discriminate between patients in real remission and patients at risk for relapse.

The same factors for epithelial integrity and chronic repair in combination with alterations in mesenchymal stroma might help to understand differences between current- and ex-smokers, and an accelerated decline in lung function. The nature of the matrix that surrounds airway smooth muscle probably plays a role in differences in decline of lung function. Comparing bronchial smooth muscle and matrix proteins as, i.e. collagens, fibronectin, elastin, tenascin, decorin, and laminin, may increase our understanding of the different courses of asthma as found in our cohorts.

Genetic studies may add to our understanding of the heterogeneity of asthma. Also in our studies, large individual differences in airway inflammation and remodeling were found, even within clinically well-defined subgroups. Genetic studies in these subgroups, like asthma in remission, progressive asthma, severe corticosteroid insensitive asthma, and asthma with systemic inflammation, may gain more insight into which genetic and environmental risk factors contribute to the course of asthma over a life span. The

current improvements in the application of genome-wide association scans, genome-wide expression, microRNA studies, and new epigenetic insights will undoubtedly result in a substantial step forward to understand the mechanisms underlying asthma(14).

Our results suggest that aiming for clinical control of asthma indeed is associated with suppression of the underlying airway inflammation. We found that PC20AMP, eosinophils, and epithelial integrity were independent factors contributing to the level of asthma control in our cross-sectional study. Randomized intervention studies with a longitudinal study design are needed to further demonstrate a more causal and specific relationship between these markers of inflammation and asthma control. The health-related quality of life in patients with controlled asthma was significantly better than in those with uncontrolled asthma. In contrast to a previous study(15)(, we found a significantly higher percentage of ICS users and dose of ICS used in the uncontrolled group. An “unhealthy ICS” effect may well have been present in our study since it is well-known that some asthmatics do not respond well to corticosteroids(16;17). Longitudinal studies could further investigate which surrogate markers for asthma control and/or airway inflammation can predict responsiveness to treatment with inhaled corticosteroids. Possibly, there may also be a role for the “electronic nose” in assessing asthma control in the future. The electronic nose analyses volatile organic compounds in exhaled breath. Recently, using 32 sensors, the electronic nose was able to discriminate patients with asthma from controls, but was less accurate in distinguishing asthma severities (18). Treatment with inhaled corticosteroids should be recommended in patients with uncontrolled asthma and combined with interventions to improve medication adherence. Which interventions are best in improving medication adherence are still not clear(19). If the use of inhaled corticosteroids can be increased in patients who are likely to respond to this treatment, we may expect a further increase in quality of life, a decrease in the number of asthma exacerbations and, possibly, also a decrease in decline of lung function.

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Nederlandse Samenvatting

HET BELOOP VAN ASTMA

Astma is een chronische ontsteking van de luchtwegen en gaat vaak gepaard met een wisselende vernauwing van de luchtwegen. Bij patiënten met (chronisch) astma kunnen de klachten leiden tot een verminderde kwaliteit van leven. De astmaklachten worden waarschijnlijk veroorzaakt door toegenomen activiteit van ontstekingscellen in de luchtwegen. Deze ontstekingscellen scheiden verschillende stoffen uit en deze zorgen o.a. voor luchtwegvernauwing en toegenomen slijmproductie. Tegelijkertijd zorgt deze chronische ontstekingsreactie voor veranderingen in de luchtwegwand en omringend weefsel zoals verlittekening, verdikking van spierweefsel en groei van nieuwe bloedvaten. Uiteindelijk kunnen deze processen leiden tot blijvende schade en continue vernauwing van de luchtwegen. Hiertegenover staat dat sommige

astmapatiënten over hun astma heen lijken te groeien en na verloop van tijd geen klachten meer aangeven terwijl ze geen astmamedicijnen meer gebruiken. Dit wordt wel remissie van astma genoemd. Of remissie van astma echt bestaat is onduidelijk.

Bij eerdere onderzoeken naar remissie van astma werd nog vaak toegenomen ontsteking in de luchtwegen gevonden. Ook reageerden de luchtwegen van deze patiënten nog vaak met luchtwegvernauwing op ademhalingstesten, dit wordt ook wel overgevoeligheid van de luchtwegen genoemd. Deze bevindingen suggereren dat bij (een deel van) deze mensen toch nog steeds astma aanwezig is. Eerder is bedacht om deze patiënten zonder klachten verder onder te verdelen in een groep met patiënten zonder medicijnen en klachten, maar wel met luchtwegafwijkingen. Dit zijn patiënten in klinische astma remissie. De andere groep heeft ook geen medicijnen en klachten, maar daarnaast ook geen functionele luchtwegafwijkingen. Deze patiënten zijn in complete astma remissie. Of complete remissie ook betekent dat er geen ontsteking van de luchtwegen meer is, werd niet eerder onderzocht.

FACTOREN DIE VAN INVLOED ZIJN BIJ HET BELOOP VAN ASTMA

Er is nog steeds weinig bekend over factoren die bepalen of astma in ernst zal toenemen of juist zal verbeteren. Het lijkt er op dat de duur van astma belangrijker is voor het ontstaan van permanente schade aan de luchtwegen dan de leeftijd van de patiënt. Eerdere studies hebben meerdere risicofactoren laten zien voor een versnelde achteruitgang in longfunctie zoals een matige longfunctie op jonge leeftijd, matige reactie op medicijnen die de vernauwing van de luchtwegen moeten tegengaan (beta-agonisten), toegenomen overgevoeligheid van de luchtwegen, toegenomen slijm productie, mannelijk geslacht en roken.

Een aantal van deze risicofactoren is ook gerelateerd aan veranderingen in de luchtwegwand en ontsteking zoals een dikkere luchtwegwand, een dikker basaal membraan, verlittekening van de luchtwegen, toename van aantal en grootte van de slijmbekercellen en het aantal (eosinofiele) ontstekingscellen in het bloed.

Aan de andere kant blijkt in lange studies dat soms tot 25% van de astmapatiënten over hun ziekte heen lijkt te groeien. Een jongere leeftijd bij het van ontstaan van astma, het hebben van een vrijwel normale longfunctie en weinig overgevoeligheid van de luchtwegen zijn geassocieerd met verdwijnen van astma en van de overgevoeligheid van de luchtwegen op latere leeftijd.

Om het beloop van astma te kunnen bepalen, is meting van longfunctie belangrijk. Bij de bepaling van de longfunctie is van belang te weten dat de longfunctie steeds toeneemt tot het bereiken van een plateaufase op ± 20 jarige leeftijd en vanaf ± 30 jaar weer afneemt. De longfunctie die we vaak gebruiken is de FEV1 waarde, te weten de hoeveelheid lucht die een astmapatiënt na volledige inademing binnen 1 seconde kan uitblazen. Dit is een maat voor de hoeveelheid vernauwing van de luchtwegen.

ONTSTEKING VAN DE LUCHTWEGEN

Astma wordt gekenmerkt door ontsteking van de luchtwegen waarbij veel cellen een rol spelen. Dit zijn o.a. epitheelcellen (binnenbekleding van de luchtwegen), endotheelcellen (binnenbekleding van bloedvaten), fibroblasten (cellen in staat tot vorming van littekenweefsel), zenuwen en verschillende typen ontstekingscellen zoals eosinofiele cellen en mestcellen. In de luchtwegen van astmapatiënten ontstaat een ontstekingsreactie, bijvoorbeeld na inademen van pollen in de lucht, die een allergische reactie oproepen. Deze ontstekingsreactie veroorzaakt uiteindelijk schade aan de luchtwegen. De allergische reactie begint over het algemeen vanuit mestcellen die geactiveerd raken door allergische prikkels. De mestcellen stoten dan allerlei stoffjes uit (o.a. histamine, prostaglandine) die op hun beurt zorgen voor luchtwegvernauwing, slijmsecretie en zwelling van de luchtwegwand. Ook eosinofiele ontstekingscellen raken hierdoor geactiveerd waardoor hun aantal toeneemt in de long. Geactiveerde eosinofiele ontstekingscellen stoten stoffen uit die in staat zijn de luchtwegwand te beschadigen. De gevoeligheid van allergische prikkels wordt mogelijk ook verhoogd door veranderingen in de epitheelcellen bij astmapatiënten. Mogelijk is de laag epitheelcellen overgevoelig voor allergische prikkels en zorgt op zichzelf via uitstoot van stoffjes (cytokinen) voor een toegenomen ontstekingsreactie. Ook is bekend dat de laag epitheelcellen in de longen van astmapatiënten niet geheel sluitend is en dat via openingen in deze laag (allergische) stoffen makkelijker de (ontstekings)cellen in de long kunnen prikkelen. De gedachte is dat door voortdurende prikkeling, het epitheel uiteindelijk terechtkomt in een continue staat van ontsteking welke uiteindelijk zorgt voor blijvende schade aan de luchtwegen. Deze schade uit zich o.a. door een veranderde samenstelling van de luchtwegwand.

VERANDERINGEN VAN DE LUCHTWEGWAND

Onderzoek bij patiënten met ernstig astma heeft aangetoond dat ernstig astma gekarakteriseerd wordt door een toegenomen dikte van de luchtwegwand in vergelijking tot milder astma. Factoren die geassocieerd worden met een dikkere luchtwegwand zijn o.a. meer slijmbekercellen, onrustige epitheelcellen, een dikker basaalmembraan (laagje weefsel onder de epitheelcellen), verlittekening, dikkere luchtwegspieren, toegenomen aantal bloedvaten en veranderingen in de kleinere luchtwegen (richting longblaasjes).

ASTMA EN ROKEN

In Nederland werd in een jonge groep astmapatiënten met een gemiddelde leeftijd van 24.7 jaar gevonden dat 33% rookte! Dit percentage is hoog en vergelijkbaar met het aantal rokers in de algemene bevolking. Het beloop van astma wordt namelijk negatief beïnvloed door roken. In vergelijking met niet rokende astmapatiënten, hebben rokers meer astmasymptomen, meer astma aanvallen, meer ziekenhuisopnames door astma, een versnelde achteruitgang van longfunctie en meer sterfte door astma. Roken zorgt ook voor meer ontsteking in de long. Deze ontsteking is qua type deels vergelijkbaar met niet rokende astmapatiënten, maar kent ook verschillen zoals een groter aantal andere typen ontstekingscellen zoals neutrofiele granulocyten en macrofagen. Belangrijk is ook dat roken bij astmapatiënten leidt tot meer veranderingen in de samenstelling van de luchtwegwand dan bij niet-rokende astmapatiënten.

Momenteel is weinig bekend over de mechanismen die verantwoordelijk zijn voor de slechte uitkomst van astma door roken. Dit komt omdat in veel studies rokende astmapatiënten worden uitgesloten omdat ze soms ook longemfyseem hebben.

BEPALEN VAN DE MATE VAN ONTSTEKING IN DE LUCHTWEGEN

De mate van ontsteking in de luchtwegen is rechtstreeks te meten door bipten (kleine hapjes) uit de luchtwegwand te nemen via een kijkonderzoek van de longen (bronchoscopie). Dit is echter voor patiënten een belastend onderzoek en daardoor niet geschikt om de behandeling van astma te sturen. Een aantal indirecte metingen

wordt gebruikt om een inschatting te maken van de hoeveelheid ontsteking van de luchtwegen.

METING VAN LUCHTWEGERGEVOELIGHEID DOOR INADEMEN VAN HISTAMINE OF METHACHOLINE

Histamine en methacholine zorgen rechtstreeks voor samentrekken van de spieren rondom astmatische luchtwegen en zorgen zo voor luchtwegvernauwing. Bij deze test wordt histamine of methacholine ingeademd door de patiënt. Begonnen wordt met een hele lage dosis en deze wordt geleidelijk verhoogd. Na elke dosis wordt de FEV₁ gemeten. Als de FEV₁ met 20% is gedaald en de luchtwegen dus fors vernauwd zijn geraakt wordt de test beëindigd. De patiënt krijgt dan een medicijn (beta-agonist) om de luchtwegen weer te verwijden. Hoe sneller de luchtwegen vernauwd raken, en dus bij een lagere dosis histamine of methacholine, hoe overgevoeliger de luchtwegen zijn. Uit eerdere studies is gebleken dat de mate van overgevoeligheid voor histamine of methacholine gerelateerd is aan de mate van ontsteking van de luchtwegen. Deze relatie is echter niet heel sterk en matig gevoelig voor veranderingen in de intensiteit van ontsteking in de luchtwegen.

METING VAN LUCHTWEGERGEVOELIGHEID DOOR INADEMEN VAN ADENOSINE-'5-MONOPHOSPHATE (AMP)

Net als met histamine of methacholine kan de overgevoeligheid van luchtwegen ook gemeten worden aan de hand van inademingstesten met AMP. Ook nu wordt begonnen met een inademen van een lage dosis en kijkt men bij welke dosis de met FEV₁ met 20% is gedaald. In tegenstelling tot histamine of methacholine werkt AMP niet rechtstreeks op de spieren rondom de luchtwegen. AMP valt na inademing uiteen tot adenosine en adenosine op zijn beurt activeert mestcellen. Een geactiveerde mestcel stoot onder andere histamine uit wat dan weer zorgt voor samentrekken van de spieren rondom astmatische luchtwegen. Uit eerder onderzoek is gebleken dat de inademingstest met AMP, beter dan de inademingstest met histamine of methacholine, de hoeveelheid ontsteking in de luchtwegen van astmapatiënten weergeeft. Ook verandert de gevoeligheid voor inademing van AMP sneller bij veranderingen in de hoeveelheid ontsteking in de luchtwegen, bijvoorbeeld na inademing van ontstekingsremmende medicijnen.

METING VAN DE HOEEVEELHEID STIKSTOF MONO-OXIDE (NO) IN DE UITADEMINGSLUCHT

Bij ontstekingsprocessen wordt NO geproduceerd. NO komt bij ontsteking in de long vrij in de uitademingslucht en kan gemeten worden. Eerdere studies hebben laten zien dat de ernst van astma samen gaat met de hoeveelheid uitgeademd NO. Ook daalt de hoeveelheid NO in de uitademingslucht na behandeling met ontstekingsremmers. Er is in eerdere studies geprobeerd de behandeling van astma met ontstekingsremmers te sturen aan de hand van de hoeveelheid NO in de uitademingslucht. Slechts 1 van de 6 studies liet hiervan positieve resultaten zien

ONTSTEKINGSCELLEN IN LUCHTWEGLIJM

Het is mogelijk ontstekingscellen te meten in opgehoest slijm uit de longen. Dit gebeurt door astmapatiënten enige minuten een nevel van zout water in te laten ademen. Dit zoute water trekt in de long ontstekingscellen aan, zodat na ophoesten deze ontstekingscellen gemeten kunnen worden. Eerdere studies hebben laten zien dat de behandeling van astma met ontstekingsremmers te sturen is aan de hand van de hoeveelheid eosinofiele ontstekingscellen in het luchtwegslijm. Deze gestuurde behandeling leidde tot minder astma-aanvallen en ziekenhuisopnames dan in de controlegroep. Ook bij onderzoek met behulp van biopten bleek de ontsteking in de

luchtwegwand duidelijk minder bij de gestuurde behandeling dan bij de controlegroep.

ASTMA CONTROLE

In de (GINA) astma richtlijnen van 2006 wordt benadrukt dat controle van de ziekte astma heel belangrijk is. Patiënten moeten zo min mogelijk astmasymptomen hebben, daarnaast geen astma-aanvallen, geen achteruitgang van longfunctie en zo weinig mogelijk bijwerkingen van de astmamedicijnen. The GOAL (Gaining Optimal Asthma control) studie in 2004 heeft met behulp van het bijhouden van dagboekjes met daarin astmaklachten, de frequentie van gebruik van luchtwegverwijders en een simpele longfunctietest, geprobeerd controle van astma te definiëren en zo de behandeling van astma te verbeteren. Als patiënten te veel astmasymptomen hadden, werd de medicatie aangepast met het doel bij zo veel mogelijk patiënten controle van astma te bereiken. Voor een groot aantal patiënten in deze studie bleek het inderdaad mogelijk om zo hun astma onder controle te krijgen.

Er zijn ook een aantal vragenlijsten beschikbaar die de mate van astma controle kunnen meten. Deze vragenlijsten zijn de asthma controle questionnaire (ACQ), de asthma related quality of life questionnaire (AQLQ) en de asthma control test (ACT). Er is niet goed bekend of meer controle van astma zoals gemeten met een vragenlijst ook samengaat met minder ontsteking van de luchtwegen. Wel is eerder een relatie gevonden tussen de mate van controle volgens de ACQ vragenlijst en de hoeveelheid eosinofiele ontstekingscellen in luchtwegslijm en overgevoeligheid van de luchtwegen voor methacholine. Andere studies vonden ook een verband tussen controle volgens astma vragenlijsten en de hoeveelheid NO in de uitademingslucht.

Tot nu toe zijn er geen studies geweest die astma controle volgens vragenlijsten hebben vergeleken met de hoeveelheid ontsteking in biopsaten uit de luchtwegwand.

ONDERZOEKSDOELEN

1. Te onderzoeken of ontsteking van de luchtwegen en veranderingen van de luchtwegwand verdwenen zijn bij (voormalig) astmapatiënten die voldoen aan de criteria voor complete astma remissie (Hoofdstuk 2 en 3).
2. Te onderzoeken of er verschillen bestaan in ontsteking van de luchtwegen en veranderingen van de luchtwegwand tussen astmapatiënten die adenosine-5'-mono phoshate (AMP) gevoelig of ongevoelig zijn (Hoofdstuk 4).
3. Te onderzoeken of er verschillen bestaan in ontsteking van de luchtwegen en veranderingen van de luchtwegwand tussen astmapatiënten in remissie en astmapatiënten met een trage en snelle achteruitgang van longfunctie (Hoofdstuk 5).
4. Te onderzoeken welke factoren in de luchtwegen een rol spelen bij schade aan de luchtwegen van astmapatiënten door roken (Hoofdstuk 6).
5. Te onderzoeken of er een relatie is tussen de (klinische) mate van astmacontrole en directe of indirecte metingen van ontsteking in de luchtwegen en veranderingen aan de luchtwegwand (Hoofdstuk 7)

RESULTATEN VAN DE STUDIES

REMISSIE VAN ASTMA

Om te onderzoeken of remissie van astma echt bestaat, hebben we onderzoek gedaan naar ontsteking in de luchtwegen en structurele veranderingen in de luchtwegwand. Dit hebben we onderzocht bij patiënten die geen ontstekingsremmende (astma)medicijnen gebruikten (of hebben gebruikt) en die nooit hebben gerookt. Een grote groep astmapatiënten die eerder onderzocht was, hebben we opnieuw opgeroepen om te zoeken naar patiënten die voldeden aan de criteria van complete astma remissie. De criteria voor complete astma remissie zijn: geen astmaklachten, geen astmamedicijnen, geen permanente vernauwing van de luchtwegen en geen overgevoelige luchtwegen. Deze groep mensen werd vergeleken met astmapatiënten die nog wel overgevoelige luchtwegen hadden. Beide groepen ondergingen de AMP ademtest. Van deze test weten we dat als we overgevoelige luchtwegen bij astmapatiënten gaan prikkelen door AMP in te laten ademen, dit leidt tot een ontstekingsreactie in de long en daarnaast tot luchtwegvernauwing.

In hoofdstuk 2 hebben we vergeleken of ontstekingscellen in luchtwegslijm ook verschilden tussen patiënten die nog steeds astma hadden en patiënten die over hun astma heengegroeid leken en voldeden aan de criteria van complete astma remissie. De ontstekingscellen werden vergeleken voor inademen van AMP en na inademen van AMP. We vonden dat er voor inademen van AMP geen verschil was in de hoeveelheid ontstekingscellen tussen de beide groepen. Echter, na het inademen van AMP zag je een toename van ontstekingscellen in de groep astmapatiënten met nog steeds overgevoelige luchtwegen, maar niet bij de patiënten met de kenmerken van complete astma remissie.

Hierin vonden we ondersteuning voor de gedachte dat bij vroegere astmapatiënten die over hun astma heengegroeid leken en daarnaast geen overgevoelige luchtwegen meer hadden, ook daadwerkelijk minder ontsteking in de luchtwegen was. Dit zou erop kunnen wijzen dat deze groep ook echt volledig over hun astma heengegroeid was.

In hoofdstuk 3 hebben we complete remissie van astma verder onderzocht. Nu hebben we ook gekeken naar ontsteking van de luchtwegwand zelf. Hiervoor hebben we via een kijkonderzoek biopten uit de luchtwegwand genomen om deze onder de microscoop te onderzoeken. We vergeleken hierbij patiënten met complete astma remissie met patiënten in klinische remissie en patiënten met blijvend astma. Ook rokende astmapatiënten deden nu mee aan deze studie. We vonden dat eosinofiele ontstekingscellen minder aanwezig waren in de luchtwegwand en in het bloed van patiënten met complete astma remissie. Zoals eerder genoemd zijn eosinofiele ontstekingscellen zeer kenmerkend voor astma en zorgen door het uitscheiden van stoffen waarschijnlijk voor (veel) schade aan de luchtwegen.

Daarnaast vonden we dat een bepaalde laag van de luchtwegwand, het basaalmembraan, net zo dik was bij astmapatiënten met complete remissie als bij patiënten met blijvend astma. Hierbij valt op dat dit kenmerk tussen deze beide groepen wel hetzelfde is, maar dat de ene groep (complete remissie) geen overgevoelige luchtwegen meer heeft en de andere groep (blijvend astma) wel. We denken dat vooral de ontsteking met eosinofiele ontstekingscellen dus bepalend is voor overgevoelige luchtwegen en dus niet zozeer de opbouw van de luchtwegwand.

AMP GEVOELIGHEID

We weten dat prikkeling van de luchtwegen door inademen van AMP zorgt voor luchtwegvernauwing bij de meeste astma patiënten. Nu zijn niet alle patiënten gevoelig voor AMP en is het nog niet goed bekend waardoor dit komt. Om te bewijzen dat onze AMP ongevoelige astmapatiënten wel degelijk astma hadden, kregen ze een extra ademtest met histamine. Van histamine weten we dat eigenlijk elke astmapatiënt reageert met luchtwegvernauwing. Dit was ook bij de AMP ongevoelige astmapatiënten in onze studies het geval.

In hoofdstuk 2 en 4 hebben we onderzoek gedaan naar de kenmerken van astmapatiënten die wel of niet gevoelig zijn voor AMP. In hoofdstuk 2 vonden we dat AMP ongevoelige astma patiënten minder eosinofiele ontstekingscellen in luchtwegslijm hadden zitten dan AMP gevoelige astmapatiënten. Dit verschil was ook significant. Daarnaast waren de AMP gevoelige astmapatiënten vaker (en meer) allergisch dan AMP ongevoelige patiënten.

In hoofdstuk 4 vonden we dat er meer eosinofiele ontstekingscellen in de luchtwegwand waren van astmapatiënten die AMP gevoelig waren dan bij astmapatiënten die AMP ongevoelig waren. Ook de basaalmembraan van de luchtwegwand was dikker bij AMP gevoelige patiënten. De mate van AMP gevoeligheid nam toe met meer eosinofiele ontstekingscellen in de luchtwegwand en daarnaast ook met een dikkere basaalmembraan. Ook tussen de hoeveelheid eosinofiele ontstekingscellen in de luchtwegwand en dikte van het basaalmembraan was een positief verband.

Dat AMP gevoeligheid samen gaat met meer eosinofiele ontsteking pleit ervoor dat we AMP gevoelige astmapatiënten misschien eerder moeten gaan behandelen met ontstekingsremmende medicijnen. Ten eerste omdat eosinofiele ontsteking samen lijkt te gaan met (blijvende?) veranderingen van de luchtwegwand. Ten tweede omdat we uit eerder onderzoek weten dat patiënten met eosinofiele ontstekingscellen vaak goed verbeteren na behandeling met ontstekingsremmers.

Aan de andere kant zagen we dat er bij AMP ongevoelige patiënten minder eosinofiele ontstekingscellen aanwezig waren en minder verdikking was van de basaal membraan. Dit zou kunnen betekenen dat AMP ongevoelige astmapatiënten misschien wel een milde vorm van astma hebben. Echter de beperkte hoeveelheid ontsteking werd niet bij elk individu uit de AMP ongevoelige groep gezien. Dat betekent dat AMP ongevoeligheid individueel dus (nog) geen betekenis heeft voor de dagelijkse praktijk.

ACHTERUITGANG VAN LONGFUNCTIE

In hoofdstuk 5 hebben we (voormalige?) astmapatiënten met complete remissie vergeleken met huidige astmapatiënten met een trage (< 30 ml/jaar) en een snelle (>30 ml/jaar) achteruitgang in longfunctie. In de groep patiënten met complete astma remissie was de achteruitgang in longfunctie gemiddeld 8 ml/jaar, bij huidige astmapatiënten met een trage achteruitgang 18.6 ml/jaar en met een snelle achteruitgang 50.3 ml/jaar.

Huidige astmapatiënten met een snelle achteruitgang in longfunctie waren gevoeliger voor AMP en hadden meer eosinofiele ontstekingscellen in bloed en luchtwegslijm in vergelijking met de twee andere groepen. Ook leken de eosinofiele ontstekingscellen actiever en hierdoor meer in staat tot de uitstoot van, voor de luchtweg, schadelijke stoffen. Opvallend was dat we in de luchtwegwand zelf geen verschil konden vinden in de hoeveelheid (en soort) ontstekingscellen tussen de drie groepen. Ook de samenstelling van de luchtwegwand was idem tussen de drie groepen.

Over alle groepen gezamenlijk, zagen we dat de mate van achteruitgang van longfunctie toenam bij meer eosinofiele ontstekingscellen in luchtwegslijm.

Mogelijk kan het ontbreken van verschillen in de mate van ontsteking in de luchtwegwand zelf tussen de groepen wordt veroorzaakt doordat (eosinofiele) ontstekingscellen rechtstreeks van het bloed naar het luchtwegslijm gaan en niet blijven hangen in de luchtwegwand. Dit benadrukt het belang van het meten van ontstekingscellen in het luchtwegslijm omdat de hoeveelheid van deze cellen in het luchtwegslijm was gekoppeld aan een toegenomen achteruitgang in longfunctie. Dus ook de bevindingen uit deze studie pleiten voor behandeling met ontstekingsremmende medicatie bij astmapatiënten met een toegenomen aantal eosinofiele ontstekingscellen in luchtwegslijm.

ASTMA EN ROKEN

In hoofdstuk 6 hebben we de effecten van roken bestudeerd bij een grote groep astmapatiënten. We vergeleken rokers met ex-rokers en nooit-rokers. In deze groepen hebben we astmasymptomen, ontsteking en veranderingen in de luchtwegwand onderzocht. We vonden een verband tussen roken en toegenomen verandering in de samenstelling van de luchtwegwand. Daarnaast was er een verband tussen toename van

veranderingen in de luchtwegwand en toename van astmaklachten zoals aangegeven door de patiënt zelf. Bij actieve rokers werden minder eosinofiele ontstekingscellen en mestcellen gevonden dan bij nooit-rokers. Dit is eerder gevonden en betekent dat andere factoren dan eosinofiele ontstekingscellen bij rokers zorgen voor de versnelde achteruitgang van longfunctie. Tot onze verrassing was het aantal mestcellen en de mate van verandering van de luchtwegwand gelijk tussen ex-rokers en nooit rokers. Dit suggereert dat schade door sigarettenrook (deels) omkeerbaar is na stoppen met roken.

Het belangrijkste verschil tussen (ex-)rokers en nooit-rokers was het toegenomen aantal eosinofiele ontstekingscellen bij nooit rokers in vergelijkingen tot beide andere groepen. Het aantal eosinofiele ontstekingscellen nam toe met duur van stoppen van roken in de groep van ex-rokers. Dit suggereert dat het onderdrukken van eosinofiele ontsteking door roken verdwijnt na stoppen met roken. Dit zou voor de patiënten gunstig kunnen zijn, omdat eosinofiele ontsteking vaak goed reageert op behandeling met ontstekingsremmers.

ASTMA CONTROLE

In hoofdstuk 7 werd onderzocht of er een verband was tussen verschillende manieren van het meten van astma controle en de gemeten mate van ontsteking in de luchtwegen en kwaliteit van leven. We vergeleken 69 patiënten die hun astma voldoende onder controle hadden (volgens de GOAL criteria) met 42 patiënten die hun astma niet onder controle hadden. We vonden dat patiënten met gecontroleerd astma minder eosinofiele ontstekingscellen hadden in het bloed, minder gevoelig waren voor AMP en minder stikstofmonoxide in de uitademingslucht hadden dan patiënten met ongecontroleerd astma. Ook in bipten van de luchtwegwand van patiënten met gecontroleerd astma vonden we minder actieve eosinofiele ontstekingscellen en minder schade aan het epitheel dan in ongecontroleerd astma.

Deze bevindingen zijn belangrijk omdat de mate van astma controle werd vastgesteld met behulp van een simpel dagboek met symptomen en een eenvoudige blaastest (GOAL criteria). Nu blijkt dat deze relatief eenvoudig uit te voeren bepaling van astma controle ook echt samen gaat met de mate van ontsteking in de luchtwegen en de hoeveelheid veranderingen van de luchtwegwand van astmapatiënten. Dit ondersteunt dus deze manier van meten van astma controle.

De gemeten kwaliteit van leven volgens de AQLQ vragenlijst was hoger bij patiënten met gecontroleerd astma dan bij de groep met ongecontroleerd astma. Dit geeft aan dat streven naar een betere astma controle met behulp van de GOAL criteria ook daadwerkelijk voor patiënt een merkbaar verschil maakt.

BELANGRIJKSTE BEVINDINGEN IN DIT PROEFSCHRIFT

1. Bij (voormalig) astmapatiënten die voldoen aan de criteria van complete astma remissie wordt er geen toename van ontstekingscellen gezien in luchtwegslijm na inademen van AMP. Bij astmapatiënten met nog steeds overgevoelige luchtwegen wordt deze toename van ontstekingscellen wel gezien (Hoofdstuk 2).
2. Bij (voormalig) astmapatiënten die voldoen aan de criteria van complete astma remissie wordt er een lager aantal eosinofiele ontstekingscellen in de luchtwegwand gemeten dan bij astmapatiënten in klinische remissie en bij astmapatiënten met nog steeds astmasymptomen (Hoofdstuk 3).
3. De dikte van de basaalmembraan is gelijk bij (voormalig) astmapatiënten die voldoen aan de criteria van complete astma remissie, astmapatiënten in klinische remissie en astmapatiënten met nog steeds astmasymptomen (Hoofdstuk 3).
4. Astmapatiënten die gevoelig zijn voor AMP hebben meereosinofiele ontstekingscellen en een dikkere basaalmembraan dan AMP ongevoelige astmapatiënten (Hoofdstuk 4).
5. Bij (voormalig) astmapatiënten die voldoen aan de criteria van complete astma remissie worden evenveel eosinofiele ontstekingscellen gevonden in luchtwegslijm en luchtwegbiopten als bij astmapatiënten met een langzame achteruitgang in longfunctie. Astmapatiënten met een snelle achteruitgang in longfunctie hadden meer eosinofiele ontstekingscellen in de luchtwegen dan deze beide andere groepen (Hoofdstuk 5).
6. Rokende astma patiënten hebben veranderingen in de luchtwegwand die gerelateerd zijn aan astmasymptomen zoals kortademigheid en hoesten (Hoofdstuk 6).
7. Astmapatiënten die met roken gestopt zijn hebben een vergelijkbare samenstelling van de luchtwegwand als astmapatiënten die nooit gerookt hebben (Hoofdstuk 6).
8. De mate van astmacontrole zoals gemeten met vragenlijsten en longfunctietesten is gerelateerd aan de hoeveelheden ontsteking in de luchtwegen zoals gemeten bij directe en indirecte testen (Hoofdstuk 7).
9. De mate van astmacontrole is gerelateerd aan de kwaliteit van leven bij astmapatiënten (Hoofdstuk 7).

Dankwoord

Na 6 jaren van noeste arbeid zit het erop. Het proefschrift is af! De uitdrukking mede mogelijk gemaakt door... is op dit proefschrift zeker van toepassing. In dit laatste hoofdstuk wil ik graag iedereen bedanken die mijn werk en daarmee het proefschrift mogelijk heeft gemaakt.

Eerst nog even dit: Een internist bij de longziekten komt dat wel goed? Net aangenomen voor de opleiding interne geneeskunde en dan onderzoek doen bij de longziekten omdat een klinische studie naar de effecten van roken bij astma zo leuk lijkt. Grote kans dat ik tot inzicht zou komen en toch longarts zou willen worden.... Uiteindelijk ben ik niet bekeerd, maar wat mij betreft is het kenmerk van de internist het brede interessegebied en daar hoort longziekten (en astma in het bijzonder) ook absoluut bij!

Beste Dirkje, mijn eerste promotor, ik wil je hartelijk danken voor de fijne samenwerking de afgelopen jaren. Je scherppte, energie en daadkracht zijn bewonderenswaardig. De gezamenlijke besprekingen en brainstormsessies heb ik zeer gewaardeerd en hebben steeds geleid tot een "strak" nieuw plan met uiteindelijk ook mooie resultaten. Ik heb een dikke 3 jaar fulltime onderzoek mogen doen, dit bleek voor deze forse klinische studies te krap om snel tot een proefschrift te komen. Daarna eiste de opleiding interne geneeskunde ook de nodige tijd op. Uiteindelijk heeft het 6 jaar geduurd voor het proefschrift naar de leescommissie ging. Af en toe heb je hiervoor het nodige geduld moeten opbrengen, maar via subtiele, diplomatieke wegen (Nick) wist je de beweging er wel steeds in te houden. Inmiddels ligt hiervan het tastbaar resultaat in handen!

Beste Wim, mijn tweede promotor, onze eerste kennismaking was al tijdens de sollicitatiegesprekken voor het promotietraject. Ik had woensdagmiddag via e-mail mijn interesse voor onderzoek kenbaar gemaakt. 2 dagen later zaten jij, Nick en ik al gezamenlijk aan tafel voor de sollicitatie. Na de verkenning beiderzijds was binnen 1 week de zaak beklonken. Ik wil je graag bedanken voor de prettige samenwerking tijdens het onderzoek. We hadden mooie klinische studies in handen waarbij zeer veel materiaal werd verzameld voor verdere analyses in het laboratorium. De gezamenlijk wil om met dit kostbare materiaal tot resultaten te komen was sterk en de uitgebreide mogelijkheden die je hiertoe gecreëerd hebt, hebben, denk ik, zeker hun vruchten afgeworpen.

Beste Nick, mijn co-promotor. De afgelopen jaren en zeker de eerste 3 hebben we intensief samengewerkt. De ochtend na mijn eerste sollicitatiemail was jij degene die me direct belde en zie waar dit toe geleid heeft! Het samenwerken met jou was zeer plezierig. De interesse voor onderzoek wist je zeker over te brengen en de ruimte die ik kreeg voor overleg en eigen inbreng heb ik zeer gewaardeerd. Ik heb bewondering voor je creativiteit van denken. Jij wist, in mijn ogen, bekende paden in een heel nieuw daglicht te zetten waarbij onverwacht nieuwe wegen voor onderzoek werden gevonden. Ook de omgang en gezelligheid buiten het onderzoek tijdens congressen en eten bij jullie thuis zijn mij warm bijgebleven. Je had af en toe de dankbare taak om als bemiddelaar te fungeren als er wat te weinig progressie vanuit mijn kant leek te zijn. Je begrip, subtiliteit en hulp hebben er zeker toe bijgedragen dat het proefschrift nu afgerond is.

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Mijn mooie mannen. Lieve Lars, Stijn en Jort: Papa zal nog steeds af en toe thuis moeten werken...maar wel een stuk minder!

Beste lezer, hier houdt het proefschrift op. Dank voor de interesse!